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IDENTIFICATION AND CHARACTERIZATION OF COMPONENTS OF THE YEAST SPLICEOSOME

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ABSTRACT OF DISSERTATION

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The Graduate School

University of Kentucky

2007

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences at the
University of Kentucky

By
Shatakshi Shreekant Pandit

Lexington, Kentucky

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The spliceosome is a complex, dynamic ribonucleoprotein (RNP) complex that undergoes numerous conformational changes during its assembly, activation, catalysis and disassembly. Defects in spliceosome assembly are thought to trigger active dissociation of faulty splicing complexes. A yeast genetic screen was performed to identify components of the putative discard pathway.

This study found that weak mutant alleles of *SPP382* suppress defects in several proteins required for spliceosome activation (Prp38p, Prp8p and Prp19p) as well as substrate mutations (weak branch point mutants). This evolutionary conserved protein had been found in both yeast and mammalian splicing complexes. However, its role in splicing had not been elucidated. This study focused on understanding the cellular role of Spp382p in splicing and particularly in the discard pathway. Spp382p was found to be essential for normal splicing and for efficient intron turnover. Since Spp382p binds Prp43p and is required for intron release in vitro, *spp382* mediated suppression of splicing factor mutations might reflect lowered Prp43p activity. In agreement with this, we find that *prp43* mutants also act as suppressors. This leads us to propose a model in which defects in spliceosome assembly, like those caused by *prp38-1,* prompt Spp382p-mediated disassembly of the defective complex via Prp43p Bolstering this theory, we find that Spp382p is specifically recruited to defective complexes lacking the 5' exon cleavage intermediate and *spp382* mutants suppress other splicing defects.

I show by stringent proteomic and two-hybrid analyses that Spp382p interacts with Cwc23p, a DnaJ-like protein present in the spliceosome and co-purified the Prp43p-DExD/H-box protein. In this study, I also show that Cwc23p is itself essential for splicing and normal intron turnover. Enhanced expression of another protein, Sqs1p, structurally related to Spp382p and also found associated with Prp43p is inhibitory to both growth and splicing. Synthetic lethal and dosage suppression studies bolster a functional linkage between Spp382p, Cwc23p, Sqs1p and Prp43p and together, the data support the existence of a

Spp382p -dependent spliceosome integrity (SPIN) complex acting to remove defective spliceosomes.

Key Words – pre-mRNA, Splicing, Spp382, Discard Pathway, Spliceosome

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CHAPTER 1

INTRODUCTION

Most eukaryotic genes have introns that must be properly removed by the splicing apparatus to produce translatable mRNA. For many genes, the cellular choice of when and where to splice a pre-mRNA is regulated by developmental or environmental cues. For instance, current estimates (based on cDNA and EST studies) indicate that more than half (50-65%) of all human genes are alternatively spliced (Hastings and Krainer 2001). Regulated splicing is required to promote much of normal developmental biology and genetic impairment of this process accounts for over 100 human genetic disease states (Philips and Cooper 2000). In all cases studied to date, the regulation of alternative splicing has been shown to occur at the level of spliceosome assembly (Valcarcel and Green 1996). The spliceosome is made up of 5 snRNAs and over 70 proteins (so assembly is a complex event) (Brow 2002). By understanding how the normal splicing apparatus assembles we hope to provide insight into the nature of the enzyme's active site, the possible steps of regulation, and, ultimately, to offer suggestions for therapeutic application.

Splicing and the Spliceosome- An overview

Pre-mRNA splicing is the nuclear process by which introns are precisely removed from cellular pol II transcripts in two transesterification steps within a large RNA-protein complex called the spliceosome. In yeast, the spliceosome is

composed of five small nuclear RNAs (snRNAs) and over 70 different proteins (Brow 2002). Each snRNP in the spliceosome consists of one or two small nuclear RNAs (snRNAs), a complement of common proteins and a subset of snRNA specific proteins. In vitro, spliceosome formation precedes the chemical steps (i.e., RNA cleavage/ ligation reactions) of splicing and involves an ordered assembly of the snRNPs on the pre-mRNA (Konarska and Sharp 1986 ; Pikielny, Rymond et al. 1986; Cheng and Abelson 1987). Though alternate models have been proposed (e.g. the holospliceosome (Stevens, Ryan et al. 2002)), the isolation of cellular U2, U5, U6 snRNP complexes (Ohi, Link et al. 2002; Wang, Hobbs et al. 2003) and the studies of co-transcriptional pre-mRNA splicing (Gornemann, Kotovic et al. 2005; Lacadie and Rosbash 2005) suggest that the in vitro studies reflect what is happening in vivo.

The first clearly defined snRNP association involves recognition of the 5' splice site and the branchpoint region by U1 in an ATP independent manner to form the commitment complex (Legrain, Seraphin et al. 1988; Rosbash and Seraphin 1991). The commitment complex is then matured by the addition of the U2 snRNP at the expense of ATP resulting in the formation of pre-spliceosome. Finally, the tri- snRNP (U4/U6.U5) is added as a single particle at the expense of ATP to form the "snRNP complete" spliceosome. Once the spliceosome has been formed it undergoes structural rearrangements to transform to a catalytically active enzyme (Brow 2002). This results in loss (or displacement) of U1 and U4 snRNAs from the complex that performs the chemistry of splicing.

Chemistry of Splicing

Pre-mRNA splicing in both yeast and metazoans proceeds via two transesterification reactions (Staley and Guthrie 1998; Stevens and Abelson 2002). In the first reaction, the 2' hydroxyl group of an internal adenosine located within the conserved branch point sequence (UACUAAC in yeast; the underlined adenosine is the catalytic residue involved in the first transesterification reaction) acts as an attacking nucleophile on the phosphate bond at 5'splice site-exon 1 junction. This results in the release of exon 1 and formation of the lariat intermediate, a structure composed of exon 2 and the circularized intron. In the next step, the 3' –OH of the free exon 1 attacks the phosphate bond at the 3' splice site – exon 2 junction. This results in ligation of the two exons and the release of the lariat intron. The excised intron is then typically degraded, the spliceosome disassembled and the mRNA exported to the cytoplasm for translation.

Sequence requirements

Pre-mRNA splicing is a conserved process in eukaryotes, from the budding yeast to humans. The consensus elements are related but not identical. In yeast, the 5'splice site is G/GUAUGU where as in mammalian consensus sequence is G/GURAGU (where R= purine residue and ***/*** is the site of cleavage). The GU dinucleotide at the 5'ss is conserved in all eukaryotic pre-mRNA. The branchpoint consensus sequence, UACUAAC is highly conserved in yeast while in mammals it is more variable (YNYURAC, where Y= pyrimidine residue, R=

purine residue and the underlined adenosine is the site of branch formation). Some mutations in the branchpoint sequences in yeast abolish splicing (Jacquier, Rodriguez et al. 1985; Jacquier and Rosbash 1986). In mammals, such mutations typically result in use of alternate branchpoint near the 3'splice site. A polypyrimidine tract is typically found downstream of the branchpoint and upstream of the 3' splice site in mammalian introns. In yeast, the spacing between the branch point and 3' is variable, ranging from 10 to 155 nucleotides (Spingola, Grate et al. 1999). While not well conserved, some yeast introns contain a short pyrimidine rich tract of 8-12 nucleotides (mostly uracil) which ends one nucleotide upstream of the 3' splice site. Unlike the case for many mammalian introns, this sequence is not required for the first catalytic step in yeast splicing (Rymond and Rosbash 1985). However, Guthrie and co-workers showed that the presence of a uridine rich tract in yeast enhances the use of the 3' splice site when present (Patterson and Guthrie 1991).

The 3' splice site consensus is YAG/ (where Y= pyrimidine residue and ***/*** is the site of cleavage) in both mammals and yeast. The 3' splice site is dispensable for the 5'cleavage and lariat formation in in-vitro (Rymond and Rosbash 1985).

Metazoans also contain a second class of introns that are spliced by the 'Minor' or 'ATAC' spliceosome (reviewed in (Tarn and Steitz 1997; Patel, McCarthy et al. 2002). The substrates differ in the consensus from the major or the abundant class of pre-mRNA in that, the 5' consensus sequence for them is –AT/ (***/*** is the site of cleavage). UAUCCUUU, the branchpoint is longer and more

tightly constrained -UCCUUAACU- and the 3' splice site is YAC (or G) (Hall and Padgett 1994). The snRNAs U4, U5 and U6 are common to both classes of spliceosomes, however the U1 and U2 from the major class is replaced with U11 and U12 snRNAs.

DExD/H-box Proteins in Splicing

The spliceosome undergoes multiple rearrangements (in terms of the snRNA- pre mRNA interactions, the snRNA –snRNA interactions and the snRNA- protein interactions) during the assembly, catalysis and disassembly events that define the spliceosome cycle. These rearrangements require ATP hydrolysis and the presence of one of the eight DExD/H proteins (*PRP5, SUB2, PRP28, PRP5, BRR2, PRP16, PRP22* and *PRP43*) found in the spliceosome (Figure 1.2).

The DExD/H proteins belong to the helicase superfamily II (SF2), the largest helicase superfamily and are defined by the presence of seven motifs (as shown in Figure 1.1). The motifs Q, 1a (Walker A motif), 1b (Walker B motif) along with motif VI are required for ATP binding and hydrolysis. Mutations in the motif III uncouple the ATP hydrolysis from the unwinding, i.e. these mutants are capable of hydrolyzing ATP and yet fail to unwind the RNA (Tanner N.K., Cordin O. et al. 2003; Linder 2006). For such mutations in the *PRP2* and *PRP43* genes, the proteins are defective in splicing, indicating that both the ATP hydrolysis and RNA unwinding activity is essential for the DExD/H box protein function (Plumpton, McGarvey et al. 1994; Martin, Schneider et al. 2002).

The DExD/H proteins are believed to remodel the spliceosome by unwinding the RNA-RNA base pairings or by disrupting the RNA- protein interactions or by doing both. Mutational analysis indicated that each protein of this set plays a unique and non-redundant role in the spliceosome cycle in vitro (Figure 1.2). However, while involvement in specific steps is clear, the experiments conducted to date have yet to reveal the details directing substrate specificity for this set of splicing factors.

Spliceosome Assembly and DExD/H proteins

The step that commits the pre-mRNA to splicing is the binding of the U1 snRNP to the pre-mRNA in an ATP independent process known as commitment complex formation. Two separate commitment complex have been defined by in-vitro gel shift experiments. The first, CC1, requires only 5' splice site to be present on the pre-mRNA, while the second CC2 requires both 5' splice site and branch-point sequences for formation *(*Seraphin and Rosbash 1989), possibly suggesting that CC1 is an intermediate in formation of the more complex and slowly migrating CC2. The CC2 band contains Mud2p and branchpoint binding protein (BBP) in addition to the U1 snRNPs. The binding of Mud2 and BBP to the branch point occurs almost simultaneously and appears to be co-operative (Berglund, Abovich et al. 1998). The U1 snRNA protein, Prp40p shows a two-

FIG. 1.1. Schematic representation of the DExD/H box helicases. The 7 motifs of the DExD box helicases are shown below. The DExH box type of helicases lack the Q motif. The motifs implicated in ATP binding are colored in red, the blue areas indicate regions that interact with the RNA and shaded regions in green are those that are important for intra-protein interactions.

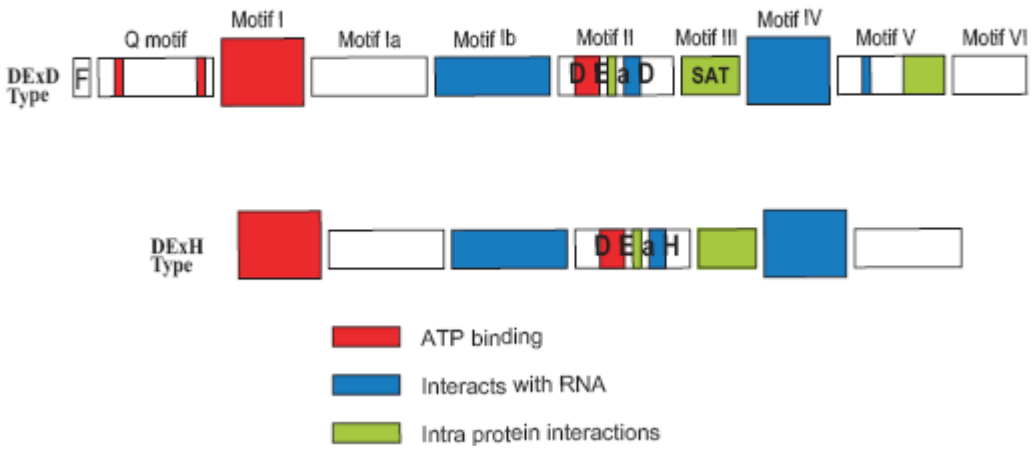
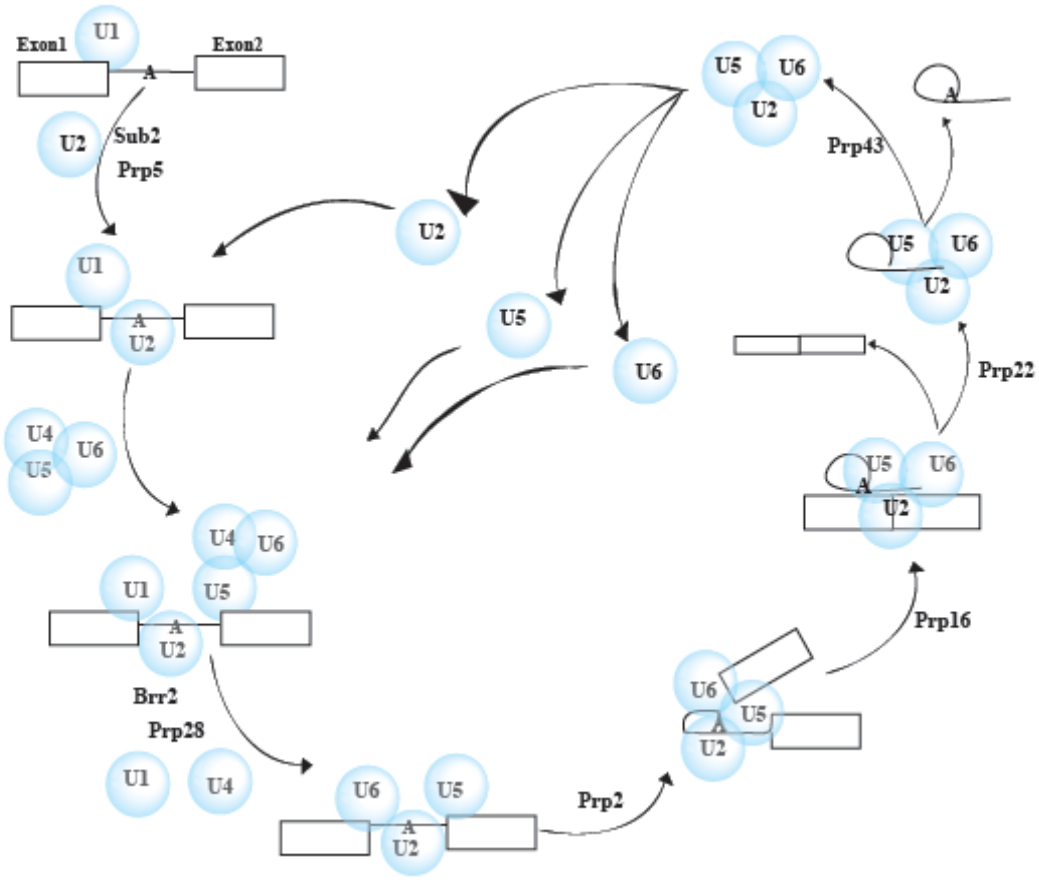


FIG. 1.2. Schematic representation of the Spliceosome assembly, catalysis and disassembly. The colored circles represent the indicated snRNAs along with their associated proteins (i.e. snRNPs) which associate with the pre-mRNA in an ordered sequence to form the spliceosome. The spliceosome then catalyzes the two transesterification reaction resulting in joining of the two exons and release of the intron as the lariat. The conformational changes that the spliceosome undergoes are believed to be bought about by the DExD/H proteins (indicated at the steps they are implicated to act) at the expense of ATP.



hybrid interaction with the branch-point protein, Bbp1p (Abovich and Rosbash 1997), thus implicating the U1 snRNP in recognition of the branchpoint. Mud2p binds BBP and with U1 snRNP protein, Prp39p and thus bridging the two ends of the intron and facilitating intron definition.

The U1 snRNA interaction with the conserved pre-mRNA sequences near the 5' splice site is stabilized by Yhc1p, the yeast homolog of the human U1C snRNP protein (Zhang and Rosbash 1999). Prp28p, a DExD/H ATPase, is implicated in the displacement of the U1 from the 5'splice site, a necessary event for later steps in spliceosome assembly involving the U6 snRNP particle. The requirement for Prp28p function can be bypassed through mutation of the *YHC1* gene or by mutation of the U1 snRNA gene to weaken it's association with 5'splice site. This result suggests that Yhc1p may be the target for Prp28p action and that displacing Yhc1p destabilizes the U1 snRNA /pre-mRNA association (Chen, Stands et al. 2001).

After formation of the commitment complex, the U2 snRNP binds to pre-mRNA in an ATP dependant manner to form the prespliceosome. Curiously, the U2 snRNA is 1000 nucleotides longer than its mammalian counterpart (Guthrie and Patterson 1988). Complementation of a yeast *snr2* (yeast U2) null allele with the human U2 snRNA showed that yeast specific U2 snRNA sequence is dispensable for growth (Igel and Ares 1988); (Shuster and Guthrie 1988). U2 snRNP binding to the pre-mRNA requires reorganization of the branchpoint region that involves, at a minimum, displacement of the BBP and Mud2 proteins. Yeast studies show that this is facilitated by two DExD/H- box proteins, Sub2p

and Prp5p (Wells and Ares 1994; Fleckner, Zhang et al. 1997; Xu, Newnham et al. 2004). The need for Sub2 protein is bypassed when *MUD2,* a non-essential gene homologous to the mammalian U2AF65, is deleted (Kistler and Guthrie 2001). This suggests that *SUB2* may promote removal of Mud2p in an ATP dependant manner. Similarly, removal of the non essential gene, Cus2p renders the U2 snRNP binding ATP independent but does not alleviate the need for the protein Prp5p (Perriman and Ares 2000; Perriman, Barta et al. 2003). This suggests that Prp5p ATPase activity is primarily needed for an internal U2 snRNP rearrangement step that involves Cus2p (Abu Dayyeh, Quan et al. 2002; Perriman and Ares 2007). Prp5p has been suggested to tether U1 and U2 snRNPs in *S. pombe* (Xu, Newnham et al. 2004). Recently, our group has provided evidence that Prp5p binds with the U2 snRNP protein Hsh155p, providing a likely U2 snRNP target in the prespliceosome formation (Wang, He et al. 2005).

The U4, U6 and U5 snRNAs are recruited to the spliceosome as the tri-snRNP particle. The U4 and U6 snRNAs are the most phylogenetically conserved snRNAs in primary sequence and size (Siliciano, Brow et al. 1987); (Brow and Guthrie 1988) and are tightly associated within this particle via extensive RNA-RNA base pairing. U4 snRNA is not essential for catalysis but acts as a chaperone for U6 snRNA. U6 snRNA plays an important role in splicing as mutations in U6 can block either the first or the second chemical step of the reaction (Fabrizio and Abelson 1990; Madhani, Bordonne et al. 1990). U6 snRNA interacts with the 5’ pre-mRNA splice site via its conserved ACAGAG sequence

(Sawa and Abelson 1992); (Lesser and Guthrie 1993; Kim and Abelson 1996). Mutations in any of these nucleotides result in temperature sensitivity or lethality (Madhani, Bordonne et al. 1990). The U6- 5' splice site interaction is prerequisite for U4-U6 unwinding (Li and Brow 1996). The first cleavage and ligation reaction occurs only after the dissociation (complete or partial) of U4 snRNA.

The U5 snRNA, in particular the conserved loop I sequence, has been crosslinked in both yeast and mammals to exon sequences near the 5' and the 3' splice site borders (Sontheimer and Steitz 1993; Newman, Teigelkamp et al. 1995). This result allows one to speculate that the role of U5 snRNA is to tether the exons in order to facilitate the second transesterification reaction. Indeed Prp8p, a core U5 snRNP protein, interacts extensively with both the 5' and the 3' splice site region prior to the second step (Teigelkamp, Newman et al. 1995; Umen and Guthrie 1996). Moreover, Prp8p mutants can suppress either 5' or 3' splice site mutations (Query and Konarska 2004). It is proposed that Prp8p functions at the catalytic core of the spliceosome by stabilizing the snRNA/pre-mRNA interactions needed for the second step of splicing (Collins and Guthrie 1999); (Grainger and Beggs 2005).

Throughout spliceosome assembly, structural rearrangements are required to configure a catalytically active enzyme. In addition to the Sub2p and Prp5p-dependent steps, these include U1 snRNA displacement from 5' splice site, dissociation of U4 snRNA from the splicing complex, formation of new base pairing between U6 and U2, and interaction of the U6 and U5 snRNAs with the pre-mRNA substrate. Brr2p is a U5 snRNP specific RNA helicase thought to

unwind U4.U6 intermolecular helices (Lin and Rossi 1996); (Kim and Rossi 1999). Brr2 has RNA dependant ATPase activity in vivo (Raghunathan and Guthrie 1998). Brr2 interacts with many other splicing factors in yeast two hybrid assay (van Nues and Beggs 2001). This suggest that Brr2 may play a role in addition to the U4/U6 snRNA unwinding. Indeed, recent evidence from the Staley lab suggests that Brr2p may play a role in both activation and disassembly of the spliceosome via the activity of the GTPase, Snu114p (Small, Leggett et al. 2006).

Several other proteins also function with the DExD/H-box proteins in spliceosome activation. For instance a mutant derivative of Prp38p (prp38-1p), a small tri-snRNP protein (28 kDa) with an acidic, serine rich C-terminus, locks the fully assembled spliceosome in an inactive state at the restrictive temperature (Blanton, Srinivasan et al. 1992);(Xie, Beickman et al. 1998) Since U4/U6 are still bound together in this mutant, Prp38p acts as a maturation factor, i.e., the spliceosome assemble without Prp38p but arrest in the pre-catalytic stage prior to U4/U6 dissociation. Related observations have been made for certain prp8p mutants (Kuhn, Reichl et al. 2002). Mutants of another U5 protein, Snu114p, a GTPase, with homology to the ribosome elongation factor EF-2, show accumulation of the splicing complex with the U4/U6 still bound together, suggesting that this GTPase protein plays a role in the unwinding (Fabrizio, Laggerbauer et al. 1997). The effector proteins, Prp38p, Prp8p or Snu114p are not helicases (based on absence of canonical helicase motifs), but may recruit or activate one such DExD/H proteins in splicing.

DExD/H box protein involvement in post-assembly events.

As discussed above, pre-mRNA splicing proceeds via two transesterification steps. Spliceosomes assemble in the absence of Prp2p, but Prp2p is required for conformational changes that promote the first cleavage – ligation reaction to generate the free upstream exon and the branched lariat intermediate (Yean and Lin 1991). While not a stable spliceosomal protein, the transient association of Prp2p accompanied by ATP hydrolysis is believed to be necessary for the formation of the catalytic core of this enzyme. Prp2p has been observed to interact directly with the pre-mRNA (Teigelkamp, McGarvey et al. 1994) but it remains uncertain how this protein is recruited to the spliceosome or how the ATPase activity might be regulated once bound. Intriguingly, Woolford and co-workers (Roy, Kim et al. 1995) identified Spp2, a yeast G-patch containing protein, in a genetic screen for dosage suppressors of the temperature sensitive *prp2* mutants. Subsequent work by Lin and co-workers (Silverman, Maeda et al. 2004) showed that the Prp2p – Spp2p interaction was necessary for the Prp2p association with the spliceosome, leading to the hypothesis that Spp2p may direct (or activate) the Prp2p DExD/H protein in the spliceosome.

Prp16p associates with the spliceosome after the first catalytic event and promotes this 3'splice site cleavage and formation of the mature message in an ATP dependant manner (Schwer and Guthrie 1992). The Guthrie lab has shown that Prp16p ATPase activity results in a conformational change that leads to protection of the 3' splice site from RNase H cleavage, and is suggested to

enhance the binding of at least 2 proteins (Prp8p and Slu7p) to the 3' splice site (Umen and Guthrie 1995).

Disassembly and DExD/H box proteins

After activation, the spliceosome performs the two transesterification steps and the intron is removed. Once the two exons are joined together, the Prp22 DExH protein promotes mRNA release (Wagner, Jankowsky et al. 1998). Prp22p has been shown to have an earlier role in splicing for transcripts where the distance between the branchpoint and the 3'SS is more than 21 nucleotides. For such splicing substrates, Prp22p depleted extracts in vitro arrest splicing after the first chemical step, resulting in the accumulation of lariat intermediates and free exon. However, unlike mRNA release, this function in promoting the second transesterification does not require ATP hydrolysis by Prp22p (Schwer and Gross 1998).

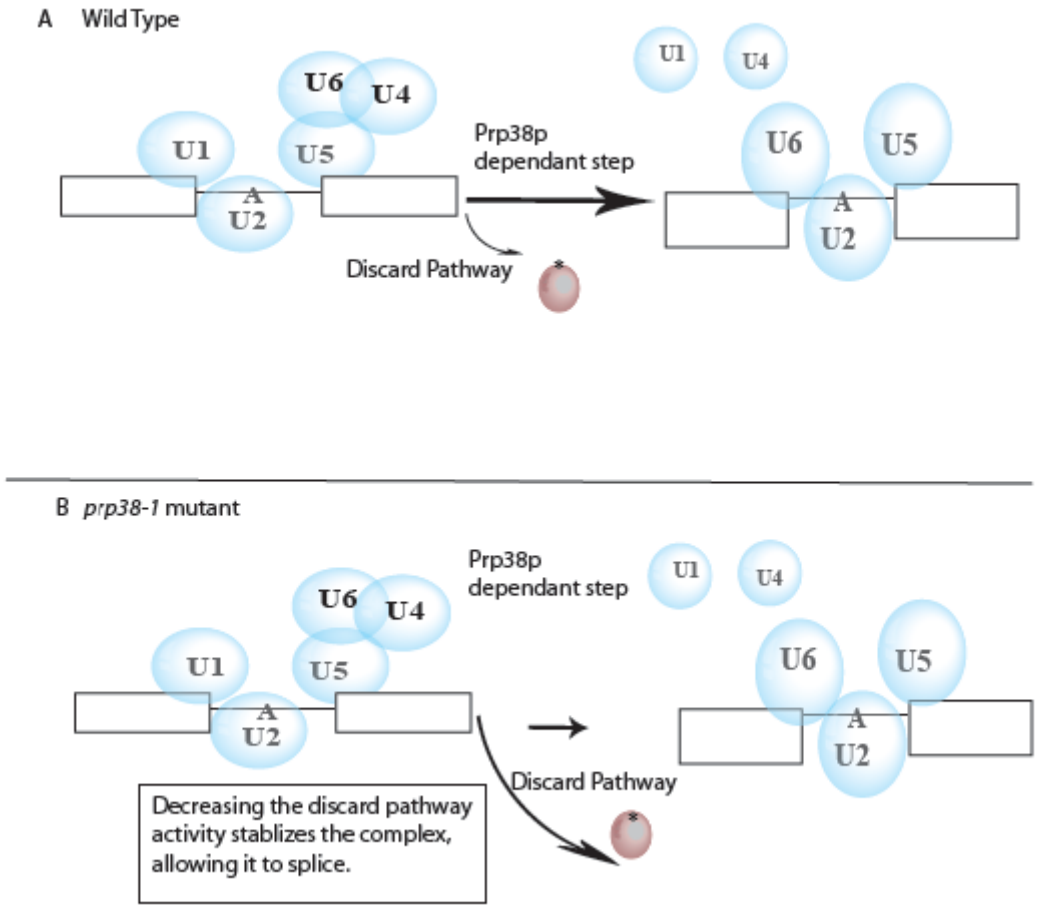
After mRNA release, the lariat intron is released from the spliceosome (through the ATPase activity of Prp43p (Arenas and Abelson 1997); (Martin, Schneider et al. 2002). The released lariat intermediate is then linearized by the 2'-5'phosphodiesterase, Dbr1p (Chapman and Boeke 1991) and subsequently degraded. The disassembled snRNPs are then recycled for a new round of splicing.

Fidelity and DExD/H box helicase

The removal of introns with single nucleotide precision is generally imperative to produce functional mRNA. Yet, how fidelity is achieved by the spliceosome is a major unanswered question in the field. Burgess and Guthrie found that a single mutation in the helicase domain of the Prp16p DExD/H box protein that diminishes its ATPase activity decreased splicing fidelity by allowing use of an aberrant branchpoint nucleotide UACUAAC→ UACUACC. They proposed a “kinetic proofreading” model where splicing fidelity was coupled to ATP hydrolysis (Couto, Tamm et al. 1987; Burgess, Couto et al. 1990; Burgess and Guthrie 1993). Similar to Prp16p, Prp22p mutants with lowered ATPase activity also allow for the use of pre-mRNA substrates aberrant 3’ss substrates (Mayas, Maita et al. 2006). Key to this “kinetic proofreading” mechanism is the predicted existence of a “discard pathway” that removes defective complexes should these persist after the step of ATP hydrolysis. It has been proposed that at each ATP driven step, the spliceosome could discard unsuitable substrates via the discard pathway (Query and Konarska 2006). Two benefits are envisioned by this activity, improved mRNA fidelity through suppression of aberrant splicing and the turnover of nonfunctional complexes, the latter being essential since splicing complexes are present in limiting amounts. Thus, while the components are unknown, such a discard pathway has been speculated to act throughout the spliceosome cycle as a quality control system for complex integrity (Konarska and Query 2005).

With this in mind, we postulated a simple hypothesis, blocked or stalled partially active spliceosomes could be suppressed either by directly enhancing their splicing efficiency or by decreasing the ability of the discard pathway to recycle them. To address this, I initiated this dissertation project with a genetic screen to identify possible effectors of the discard pathway. Rather than using a substrate-specific selection approach similar to that which identified *PRP16,* we chose to use a mutation within a core spliceosomal protein that is required for pre-mRNA splicing. This approach was reasoned to be much more stringent and less likely to identify substrate-specific suppressors. We opted to use *prp*38-*1*, a mutation in an essential component of the U4/U6.U5 tri-snRNP particle identified by our laboratory (Blanton, Srinivasan et al. 1992)). This mutation results in the assembly of kinetically impaired spliceosomes that appear to assemble normally but splice at a much reduced rate that is exacerbated by elevated temperature (Xie, Beickman et al. 1998) resulting in a tight, temperature sensitive growth (and splicing) defect. I reasoned that suppressors of *prp38-1* might occur through increased mRNA synthesis brought about by either direct improvement in splicing efficiency or by reduced activity of the proposed discard pathway – thereby providing “more time” for the defective spliceosomes to process mRNA before dissociation. This model is illustrated in figure 1.3.

FIG. 1.3. **Hypothetical model for the screen for the discard pathway.** In a simple model of splicing, the pre-mRNA is either spliced or discarded. One can speculate that under normal conditions, the rate of splicing is far greater than the rate of spliceosome disassembly and pre-mRNA degradation (Panel A as would be the case in a normal wild type cell). However when the spliceosome is blocked or stalled, as shown for *prp38-1 (*panel B*)*, it is likely to be disassembled and the pre-mRNA degraded. By extension, increased spliceosome stability is expected to increase the likelihood of processing by a partially active complex.



CHAPTER 2

MATERIALS AND METHODS

Yeast genetics and plasmid construction.

Yeast Strains

Strain	***Genotype***
MGD353-46D	*α, ura3-52, trp1-289, leu2-112,113, his*
Ts192	*α, ura3-52, trp1-289, leu2-112,113, his, prp38-1*
prp38-1	Mat *A, prp38-1, ura3-52, trp1-289, leu2-112, 113, his3, arg4*
SPM301	*α, ura3-52, trp1-289, leu2-112,113, his, prp38-1,spp382-1*
MGD353-13D	*Mat A, trp1-289, ura3-53, leu2-3,112, arg4, ade2*
Prp38::KAN, YCp50-PRP38	*MatA, ura, leu2, his, prp38::KAN, YCp50-PRP38 -URA3*
Spp382-1	*α, his3Δ1,leu2Δ0,ura3Δ0,spp382::KAN, YcpLac111-spp382-2 -LEU2)*
Spp382-2	*α, his3Δ1,leu2Δ0,ura3Δ0,spp382::KAN, YcpLac111-spp382-2-LEU2*
Spp382-3	*α, his3Δ1,leu2Δ0,ura3Δ0,spp382::KAN, YcpLac111-spp382-3 -LEU2*
Spp382-4	*A/α, his3Δ1, leu2Δ0, ura3Δ0, spp382::KAN, p416- Δspp382- URA3*

Spp382-5	*α, his3Δ1,leu2Δ0,ura3Δ0 , spp382::KAN, YcpLac111-spp382-5 - LEU2*
Spp382-6	*A/α, his3Δ1,leu2Δ0,ura3Δ0 , spp382::KAN, YcpLac111-spp382-6 - LEU2* *A, his3Δ1,leu2Δ0,ura3Δ0,CLF1-TAP-HIS, spp382::KAN, YcpLac111-spp382-6- LEU2*
Spp382-7	*α, his3Δ1, leu2Δ0, ura3Δ0, trp1-289, spp382::KAN, YcpLac111-Spp382-7 - LEU2*
Spp382-8	*A, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, spp382::KAN, YcpLac111-Spp382-8 - LEU2*
Spp382-9	*α, his3Δ1,leu2Δ0,ura3Δ0,spp382::KAN, YcpLac111-Spp382-9 - LEU2*
GAL1::*SPP382*	*α, his3Δ1, leu2Δ0, ura3Δ0, spp382::KAN, pbm150-SPP382 – URA3*
Spp382-TAP	*A, , his3Δ1, leu2Δ0, ura3Δ0, met15Δ0* Spp382-TAP
JM640	*A, prp8-1(G2347D),his7, ura1, lys2, ade1, ade2, tyr1*
JM847	*A, prp8-2 (P1015S),his7, ura1, lys2, ade1*
prp43-R242A	*α ura3-52, trp1-63, his3 Δ200, leu2-1, ade2-101, ade2-101, lys2-801, prp43::KAN, p358-prp43-R242A – TRP1*
prp43-H218A	*α ura3-52, trp1-63, his3 Δ200, leu2-1, ade2-101, ade2-101, lys2-801, prp43::KAN, p358-prp43-H218A-TRP1*

prp43-S247A	*α ura3-52, trp1-63, his3 Δ200, leu2-1, ade2-101, ade2-101, lys2-801, prp43::KAN, p358-prp43-S247A –TRP1*
prp43-T249A	*α ura3-52, trp1-63, his3 Δ200, leu2-1, ade2-101, ade2-101, lys2-801, prp43::KAN, p358-prp43-T249A- TRP1*
PJ69-4a	*a trp1-901 leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1–HIS3, GAL2–ADE2, met2 : : GAL7–lacZ*
SPC1	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, pGAL1::CWC23 - URA3*
Cwc23-1	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, YCpLa22-cwc23-1- TRP1*
Cwc23-2	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, YCpLa22-cwc23-2 - TRP1*
Cwc23-3	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, YCpLa22-cwc23-3 - TRP1*
Cwc23-4	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, YCpLa22-cwc23-4 - TRP1*
Cwc23-5	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, YCpLa22-cwc23-5 - TRP1*
Cwc23-6	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, YCpLa22-cwc23-6 - TRP1*
Cwc23-7	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, YCpLa22-cwc23-7 - TRP1*
Cwc23-8	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, pGAL1::CWC23, YCpLa22-cwc23-8 - TRP1*

Cwc23-9	*A/α, his3Δ1,leu2Δ0,ura3Δ0, trp1-289, cwc23::KAN, pGAL1::CWC23, YCpLa22-cwc23-9 - TRP1*
Cwc23-TAP	*A, , his3Δ1, leu2Δ0, ura3Δ0,met15Δ0* Cwc23-TAP
Sqs1::KAN	*A , his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, sqs1::KAN*

Microbial Methods

E.coli Methods

E. coli was cultured in 2 x YT medium (1.6% bacto tryptone, 1% bacto yeast extract, 0.5% NaCl, pH 7.5) at 37 C with ampicillin (@ 100µg/ mL) added when necessary. The strain used for sub cloning and plasmid DNA purification was TG1. The cells were made competent by the calcium chloride treatment and alkaline lysis was used for small-scale (miniprep) isolation of DNA (Sambrook K. J., Fritsch E. f. et al. 1989).

Yeast Methods

Yeast cells were routinely cultured at 25 °C in either rich media (1% bacto yeast extract, 2% bacto peptone, 2% sugar (glucose or galactose) or in selective drop out media as indicated. Yeast transformation was done by the lithium acetate method of Ito et al. (Ito, H., Fukuda, Y. et. Al. 1983) with the isolation of transformants on selective media (Sherman F., Finks G. R. et al. 1986).

For generating double mutants, the indicated strains were crossed and the diploid selected on a drop out medium that selects against the individual haploid parents. Then meiosis was induced by growth on nitrogen-limited sporulation medium. The tetrads were dissected using an Olympus BH2 microscope fitted with a micromanipulator as described by Sherman et al (Sherman F., Finks G. R. et al. 1986). The meiotic offspring from at least 20 tetrads were scored for genotypic markers on drop out media and for mating type by crossing to tester strains (see Strain List) and selecting for the resultant diploid on selective drop out media. The presence of specific chromosomal KAN^R knockouts was checked by growth in YPD with G418 (concentration of 200 mg/liter) and by direct PCR analysis of the mutant allele. For other mutants, the defective allele was confirmed by sequencing the PCR product, scoring for a restriction site polymorphism, and by complementation with the corresponding wild type allele. Mutants were checked for altered growth by streaking cultures at 19, 23, 30, 35 and 37 °C along with controll mutant and wildtype strains.

Yeast two-hybrid analyses were conducted by insertion of full-length open reading frame (ORF) PCR fragments (See Primer List) into the *GAL4* activation domain plasmid pACT and the *GAL4* DNA binding plasmid pAS2 (Clontech Laboratories Inc., Palo Alto, CA). For all the clones, except *PRP43,* PCR products were digested with *BamH1* and ligated to the pAS2 (BamH1 digested) or pACT (BglII digested) vectors. For the pAS2-*PRP43* construct, the YCpLac33-*PRP43* subclone was digested with *Xba1/Pst1* and the *PRP43* containing fragment and isolated from an agarose gel using a Qiagen DNA Purification Kit.

This DNA was then ligated to a pAS2 *Xba1/Pst1* digested vector. For all constructs, reporter gene transactivation was scored in yeast strain PJ69-4A. Unless otherwise noted, two hybrid growth results were compared after 3 days growth at 30°C on medium lacking adenine and on histidine-deficient medium supplemented with 5 or 20 mM 3-aminotriazole as described previously (James, Halladay et al. 1996).

For the checking the cis suppression, the *spp382-1*and *spp382-6* transformed with the reporter plasmids were patched and grown on a minimal plate lacking uracil at room temperature for two days. After two days, a 5 ml solution of X-Gal agar solution was overlayed onto the plate as reported in CSH Manual with the Magnesium and Phosphate concentrations adjusted to 5X the standard plate concentration to allow for the normal diffusion of the 5 ml overlay buffer/salts mix to the rest of the plate. The plates were incubated at room temperature for 16 hours and scanned.

Yeast Suppressor Screen

Spontaneous suppressors of the *prp38-1* mutant strain, ts192 (Blanton, Srinivasan et al. 1992) were selected by growth at 37 °C on rich medium (YPD (Chen, Frand et al. 1998)). Colonies were backcrossed to the wildtype strain, MGD353-13D, sporulated, and the resultant offspring scored for the reappearance of temperature sensitivity expected when the mutation is due to an extragenic suppressor. To test for dominance, the isolated suppressors were

mated to the *prp38-1* haploid yeast strain SP101 and the diploid assayed for growth at 37 °C.

The wildtype *SPP382* allele was isolated by transforming SPM301 with a centromeric yeast DNA library (Rose, Novick et al. 1987) and screening replica plates of the transformants for the re-acquisition of temperature sensitivity. A PCR and sub-cloning approach (vector YCplac33 (Gietz and Sugino 1988)) was used to identify *SPP382* as the relevant gene. (See Primer List for primer sequences for Ylr424w-1 and Ylr4242w-4).

Linkage analysis was used to confirm that the recovered gene, *SPP382*, was the wildtype allele of the original mutation and not an unlinked low dose suppressor. A PCR product containing the *SPP382* orf and 500 base pairs of upstream sequence was cloned into the *BamH1* site of YIpLac211, an integration vector. The resultant plasmid was cut at a unique site (*Hpa1*) within the insert to direct integration to homologous chromosomal locus. The parent suppressor strain, SPM301, was transformed with the linearized DNA resulting in a Ura+, temperature sensitive strain. The site of integration was checked by Southern blot analysis using radiolabeled *SPP382* gene fragment as a probe. This transformed strain was mated to a wild type (MGD353-13D) and the resulting diploid sporulated. Meiotic progeny from 21 tetrads were scored for temperature sensitivity and segregation of the *URA3* marker.

The wildtype allele of a second suppressor, *AAR2*, was identified by complementation of SPE401 using 133 genes related to RNA processing (Listed in Table 1) selected from an ordered set of yeast genes expressed with a *GAL1*

promoter. Each transformant was then screened for temperature sensitivity. Added support that the recovered gene encodes the suppressor allele was made by DNA sequence analysis of the mutant allele.

Mutagenesis

Site directed mutagenesis was done by inverse PCR with mutagenic oligonucleotides (sequences in the Primer List) and the Expand Long polymerase (Roche Biochem). The target genes were (a) the *SPP382* orf plus 450 bp of 5' flanking sequence (amplified using primers Ylr424w-1 and Ylr24w-2, see Primer list for Spp382) subcloned into the *BamH1* site of the plasmid,YCPlac111 (Gietz and Sugino 1988) and (b) the *CWC23* orf plus 100 bp of 5' and 100 bp of 3' flanking sequence (amplified using primers cwc23-1 and cwc23-2, see Primer list for the rest) cloned into BamH1 site of YCpLac22 (58). Random mutagenesis of the *CWC23* orf was done with the Mutazyme enzyme (Stratagene) and ORF specific primers (See Primer List). The mutated *CWC23* orf was used as a mega primer to amplify a full length YCpLac22-CWC23 plasmid following the manufacturer's protocol (Stratagene). In all cases, plasmids containing the mutated gene were transformed into yeast where the corresponding chromosomal allele was replaced with the Kanamycin resistance gene and a second plasmid was present bearing a wild type allele linked to *URA3* selectable marker. After isolation of the double transformant, the wild type copy was shuttled out by growing the strain on 5 flurooratic acid (Bender and Pringle 1991).

The mutagenized gene was then scored for lethality (failure to grow on the FOA plates) and for temperature or cold sensitivity at 19, 25, 30, 35 and 37 C.

For generating the *GAL1::spp382-4* promoter fusion strain, *SPP382* was amplified with primers Ylr424w-1 and Ylr424w-4 (see Primer List below), the PCR product was digested with *Cla*1, gel purified and blunt ended with T4 DNA Polymerase (New England Biolabs), phosphorylated, and then ligated into the Sma1 site of the *GAL1* yeast expression vector, p416 (Mumberg, Muller et al. 1994). The plasmid was transformed into a *spp382::KAN / SPP382* heterozygous diploid yeast strain the desired haploid strain obtained after sporulation as described above.

RNA Methods.

***Northern Analysis*:** Total cellular RNA was recovered from yeast grown under permissive or non-permissive conditions (for temperature or sugar source) and assayed by northern blot from a 1% agarose/formaldehyde gel as described by Sherman et. al (Methods in Yeast Genetics). The snRNAs were resolved on a 5% polyacrylamide, 7M urea denaturing gel and electroblotted to a Nytran membrane prior to hybridization. Uniformly radiolabeled ^{32}P probes were made with the indicated templates using the Random Primer labeling kit (Invitrogen) following the manufacturer's protocol. The RNA bands were visualized with Typhoon 8600 phosphorimager (GE Biosystems) and, where, indicated quantified with ImageQuant software.

Primer Extension Analysis: The yeast strains transformed with the intron-bearing *GAL1-RPS17* fusion gene on plasmid HZ18 (Teem and Rosbash 1983) were cultured in media lacking uracil with galactose as the carbon source. Total RNA was isolated as described above. A 5' end labeled oligonucleotide complementary to exon 2 (RB1 (see primer list), (Teem and Rosbash 1983) was used for primer extension with reverse transcriptase (Promega) as described by Rymond et al (Rymond, Pikielny et al. 1990). The cDNA products were resolved on a 5% polyacrylamide, 7M urea denaturing gel and visualized with the Typhoon 8600 phosophoimager.

***In Vitro Splicing Assay and Spliceosome Assembly*:** Splicing reactions and spliceosome assembly were performed essentially as previously described (Pikielny, Rymond et al. 1986). Splicing reactions were assembled on uniformly radiolabeled pre-mRNA prepared from *EcoRI* cleaved *SPRP51A* DNA (renamed *RPS17A*) by in vitro transcription using SP6 RNA polymerase. The reactions were resolved on 5% polyacrylamide, 7M urea denaturing gel gels and the bands were visualized using the Typhoon 8600 phosphorimager.

***Co-immunoprecipitation of snRNAs*:** 20 µl of the SPP382-Tap whole cell extract (see below) was mixed in a siliconized microfuge tube with 150 µl of buffer A [150 mM KCl, 2 mM $Mg(OAc)_2$, 50 mM HEPES, pH 7.5] and 20 µl of rabbit IgG agarose beads (Sigma) for 2 hours at 4^oC. The beads were recovered by brief low-speed centrifugation (2,000 RPM, 1 minute), and washed 6 times with 800 µl of the Buffer A. The snRNAs were then released by the addition of an equal volume of 2× PK buffer (200 mM Tris-HCl pH 7.5, 25 mM EDTA, 300 mM

NaCl, 2% SDS, 2 mg/ml proteinase K) and incubating for 10 min at 37°C. The samples were then phenol extracted, the snRNAs ethanol precipitated and resolved on a 5% polyacrylamide, 7M urea denaturing gel which was blotted and hybridized as indicated above.

SnRNP analysis: The native snRNP complexes present in yeast splicing extracts were resolved on 4% polyacrylamide (80:1 acrylamide::bisacrylamide)-0.5% agarose gels run in 50 mM Tris-glycine buffer (pH 8.8) overnight at a 30-V constant voltage and then electroblotted to a Nytran membrane. The snRNPs were visualized by hybridizing the membrane with snRNA specific radiolabeled probes.

Protein Purification and Mass Analysis.

Splicing extracts were prepared under liquid nitrogen with a Spex Certiprep 6850 freezer mill as published (Wang, He et al. 2005). TAP- tagged Spp382 was purified from 20 liters of cells by IgG agarose and calmodulin agarose affinity selection using standard procedures (Puig, Caspary et al. 2001) with NaCl increased to 450 mM in the binding and wash steps. The proteins released from calmodulin agarose were then resolved by two dimensional liquid chromatography followed by mass analysis with a Deca mass spectrometer (ThermoFinnigan) as described (Wang, Hobbs et al. 2003). The mass-intensity lists were screened against the non-redundant NCBI protein database with MASCOT software at the default cutoff score of 20.

In vivo labeling and TAP purifications

Yeast proteins were ^{35}S labeled by the addition of 1 mCi of Trans-^{35}S (ICN) to 1 ml of yeast concentrated from 10 ml of culture (at an optical density at 600 nm of 1.0) grown in low sulfur medium. The cells were incubated with the labeled amino acids at 30°C for 3 hr and harvested as described in Wang et al (Wang, Hobbs et al. 2003). TAP purification was then performed scaled down for use with the reduced volumes as recently described (Wang and Rymond 2003). Where indicated, the NaCl concentration was adjusted to 150, 300, or 500 mM during the binding and wash steps of protein A agarose chromatography. Proteins were precipitated with 6% trichloroacetic acid and the samples resolved on a sodium dodecyl sulfate-5 to 10% gradient polyacrylamide gel with Benchmark molecular weight markers (Invitrogen). The labeled bands were visualized with a Typhoon 8600 phosphorimager.

Western blotting

Western transfers of the TAP extracts on nitrocellulose membrane were blocked for half an hour in 5% milk (in PBS) and then incubated for 2 hours with a 1:1,000 dilution of rabbit anti-peroxidase primary antibody (Sigma) followed by incubation for 1 hour with a 1:1,000 dilution of goat anti-rabbit immunoglobulin G (IgG) (heavy-plus-light chain)-alkaline phosphatase secondary antibody (Gibco/BRL). The membranes were then developed with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium according to the supplier's recommendation.

Spp382p depletion

To deplete Spp382p in vivo, *GAL1*::*spp382-4* yeast strain was grown at 25°C in YP broth with 2% galactose to an optical density at 600 nm of approximately 0.20. The yeast were then harvested by centrifugation and resuspended in 2x the original culture volume with YP broth containing 2% glucose. The culture was incubated at 37°C for 16 h prior to extraction of RNA or the preparation of yeast whole cell extract as described above.

Primer List For Spp382:

Ylr424w-1	*CGG GAT CC*C AAT GCC TTCCTT TAA AAT	~450 bp upstream
Ylr424w -2	*GCG GAT CC*T GAG GTC AAG GGC CCA TAA ATA	-stop codon, end of gene.
Ylr424w -3	*GGA TCC* ATG GAG GAT TCG GAC TCC AAC	@ start site, for *GAL1* construct
Ylr424w -4	*GGA TCC* TAG AGG TCA AGG GCC CAT AA	@ end of gene
Spp382dn	TTG GAA ACA TTT CAA GGG CAA	50 bp downstream
Ylr424w -5	TAG TAC AAC CGA GAG AGG TCG	50 bp upstream
Ylr424w -6	CCC GAT AAA ACT TAG CAC TTG	Middle → start
Ylr424w -7	ATC GAC AGC GCT ATC ACC CTA	middle→ end
Ylr424w -8	*CCG GAT CCT C*AT GGA GGA TTC GGA CTC CAA	Forward for two hybrid
Ylr424w -9	GTT TTA GGC GAA GCG TTG TTA	Middle to end
Ylr424w -10	GTC ATA TCA AAC CCT TCC CAA AA	Middle to start
Ylr424w -11	AAG ATT GGT GCT ATT TTA AAA	middle→ end
Ylr424w 12	GCT CGA GTA TCT ATC CGA TTG	Middle → start
Ylr424w -13	AAA GAC TTA ACC TCC AAT GAA	For allele detection
Ylr424w -14	GAA GAC TTA ACC TCC AAT GAA	For allele detection

Primer List For The Rest:

Cwc23-1	CCC *GGA TCC* TC ATG CCA GGA CAC GAA TTG	Forward for two hybrid
Cwc23-2	CTA *GGA TC C*TA GTC GAG TAG GAT AGG TTC	Reverse for two hybrid
Cwc23-up	CCC *AAG CTT* GCA TAT TAT CTC GCA	100 bp upstream
Cwc23-dn	GGC *GGA TCC* TTT CCT TTT CTT TTT CTT	100 bp down stream
Prp43-1	ATC A*AG ATC T*CA ATG GGT TCC AAA AGA AGA TTC	Forward for two hybrid
Prp43-2	CTA T*AG ATC T*CT ATT TCT TGG AGT GCT TAC TC	Reverse for two hybrid
Ynl224c-1	CCC *GGA TCC* TAA TGG CAA AAA GGC ATA	Forward for two hybrid
Ynl224c-2	CCC *GGA TCC* GTG TAT ATT ACC ATA GAT	Reverse for two hybrid
Aar2-1	CCC *GAA TTC* GAA TGG TAA ACA TTT GGT	400 bp upstream
Aar2-2	CCC *AAG CTT* GAT ACA TCC CTT AAC CAT	200 bp down stream
Aar2-3	CCC *GGA TCC* TAA TGA ATA CTG TAC CAT TT	Forward for two hybrid
Aar2-4	CCC *GGA TCC* TTA TGG CCT TTG GTA ATA	Reverse for two hybrid
RB1	CGC TTG ACG GTC TTG GTT C	For primer extensions

Site Directed Mutagenesis Primer List:

Spp382-3: ANN GTT GCG GGA AAA GGC CTT Spp382-4: TCC CAT ACT CGA AAG TAA CTT
Spp382-A2: GCT GCA GCT ATT GAA GCA AAA TTT Spp382-A2R: AAT TGT GTC TTT GAT CTT ATC GTC
Spp382-A3: GCT GCA GCT TTC GAT TTG AGA CAG Spp382-A3R: TAA TGT AGA TTT ATC AAG GTG GTA
Spp382-L232: GCC CTT TCT GAA TTG GAG GTT ACT Spp382-L232R: CCT CTC TTG TCC ATA ATA TTC TTT
Cwc23-H1: CAA CCT GAC AAA CAC CCG GAC
Cwc23-H2: CAG CCG GAC AAT CCA TCA ATT Cwc23-H2r: TTT GTC AGG ATG ATA CTT CAG
Cwc23-H3: CAA CCT GAT TTA TTG CAA ATC Cwc23-H3R: TGG TGT GGT GGT GGG TAT CGT
Cwc23-F65: TTA CAC TTA TTA TCG ACC GCA Cwc23-F65R: TTT GTG TAT AAT TGA TGG ATT
Cwc23-A1: GCG GCC GCT AAA TAC AGG ACC CTT Cwc23-A1R: TTG GGG CAA ATC ATC GTA GAT GGT
Cwc23-A2: GCG GCC GCT AAA CAC CCG GAC AAT Cwc23-A2R: ATA CTT CAG GGC AAG GGT CCT GTA
Cwc23-A3: GCG GCC GCT AAT AAT AAA TCA AAC Cwc23-A3R: GAA GTT GTC AAG GAC AAT TCT CAA

List of splicing factors used for over expression.

No	Gene	ORF
1	AAR2	YBL074C
2	BRR1	YPR057W
3	CBC2	YPL178W
4	CDC40	YDR364C
5	CEF1	YMR213W
6	CLF1	YLR117C
7	CUS1	YMR240C
8	CUS2	YNL286W
9	CWC2	YDL209C
10	CWC15	YDR163W
11	CWC22	YGR278W
12	CWC25	YNL245C
13	DIB1	YPR082C
14	ECM2	YBR065C
15	EXO84	YBR102C
16	HSH49	YOR319W
17	GSP1	YLR293C
18	IST3	YIR005W
19	ISY1	YJR050W
20	LEA1	YPL213W
21	LSM2	YBL026W
22	LSM3	YLR438C-A
23	LSM4	YER112W
24	LSM5	YER146W
25	NAB2	YGL122C
26	LSM7	YNL147W
27	LSM8	YJR022W
28	NTF2	YER009W
29	LUC7	YDL087C
30	MER1	YNL210W
31	MSL1	YIR009W
32	MSL5	YLR116W
33	MUD1	YBR119W
34	MUD2	YKL074C
35	NAM8	YHR086W
36	NTC20	YBR188C
37	PRP2	YNR011C
38	PRP3	YDR473C
39	PRP4	YPR178W
40	PRP5	YBR237W
41	PRP6	YBR055C
42	PRP8	YHR165C
43	PRP9	YDL030W
44	PRP11	YDL043C
45	PRP16	YKR086W
46	PRP19	YLL036C
47	PRP21	YJL203W
48	PRP22	YER013W
49	PRP24	YMR268C
50	PRP28	YDR243C
51	PRP31	YGR091W
52	PRP38	YGR075C
53	PRP39	YML046W
54	PRP40	YKL012W
55	PRP42	YDR235W
56	PRP43	YGL120C
57	PRP46	YPL151C
58	RDS3	YPR094W
59	RPL30	YGL030W
60	SAD1	YFR005C
61	SKY1	YMR216C
62	SLU7	YDR088C
63	SMB1	YER029C
64	SMD1	YGR074W
65	SMD2	YLR275W
66	SMD3	YLR147C
67	SME1	YOR159C
68	SMX2	YFL017W-A
69	SMX3	YPR182W
70	SNP1	YIL061C
71	SNT309	YPR101W
72	SNU13	YEL026W
73	SNU56	YDR240C
74	SNU66	YOR308C
75	SNU71	YGR013W
76	SNU114	YKL173W
77	SPP2	YOR148C
78	SPP381	YBR152W
79	SPP382	YLR424W
80	SUB2	YDL084W
81	SYF1	YDR416W

82	SYF2	YGR129W
83	USA1	YML029W
84	YHC1	YLR298C
85	YJU2	YKL095W
86	NTR2	YKR022C
87		YLR132C
88	LSM1	YJL124C
89	CWC23	YGL128C
90	BUD13	YGL174W
91	BuD31	YCR063W
92	PML1	YLR016C
93	GCN2	YDR283C
94	CDC33	YOL139C
95	DHH1	YDL160C
96	HTA1	YDR225W
97	IMG1	YCR046C
98	MRP7	YNL005C
99	MRP13	YGR084C
100	RVB2	YPL235W
101	KRS1	YDR037W
102	TOS4	YLR183C
103	ERB1	YMR049C
104	LIN1	YHR156C
105	UTP21	YLR0409C
106	YMC6	YML025C
107	NPL3	YDR432W
108	SRB2	YHR041C
109	YEF3	YLR249W
110	TIF4631	YGR162W
111	TIF4632	YGL049C
112	BUR2	YLR226W
113	KAP95	YLR347C
114	MSH4	YFL003C
115	NAB3	YPL190C
116	NRD1	YNL251C
117	SCP160	YJL080C
118	SGV1	YPR161C
119	SRO9	YCL037
120	SRP1	YNL189W
121	AIR2	YDL175C

122	YRA2	YKL214C
123	NAB6	YML117W
124	NUG1	YER006W
126	SQS1	YNL224C
127	ASC1	YMR116C
128	DCP2	YNL118C
129	HRT2	YOL133W
130	MEX67	YPL169C
131	RIF2	YLR453C
132	GLE2	YER107C
133	CSE1	YGL238W

CHAPTER 3

RESULTS

Identifying extragenic suppressors of the prp38-1 spliceosome maturation mutant.

Spontaneous suppressors of the *prp38-1* ts growth defect were selected on YPD medium at the non-permissive temperature of 37 °C. Spontaneous suppressors were found at a rate of roughly one per 1 X 10^6 cells plated. In total, 101 yeast strains were isolated that formed colonies after 2-3 days at 37 °C.

In principle, suppressor strains might harbor mutations within *prp38-1* (i.e., intragenic suppressors) or within another gene (i.e., extragenic suppressors). To define the subset of extragenic suppressors each strain was backcrossed to wild type yeast and the resulting meiotic offspring scored for temperature sensitivity. Unlinked extragenic suppressors will segregate from the original *prp38-1* allele and, as a consequence, ts mutants reappear in the meiotic offspring. Of the 101 suppressors analyzed this way, 11 are extragenic and the remaining 90 most likely represent second site mutations within (or reversion of) *prp38-1*.

Extragenic suppressor mutations might result from loss of function and be recessive in nature or from gain of function and be dominant alleles. To distinguish between these possibilities, the extragenic suppressor set was mated to a strain of opposite mating type bearing the original *prp*38-*1* mutation (see strain list in Materials and Methods). The resultant diploid strains are homozygous for the *prp3*8-*1* mutation and heterozygous at the suppressor locus. Each strain was tested for growth at the restrictive temperature. If the suppressor

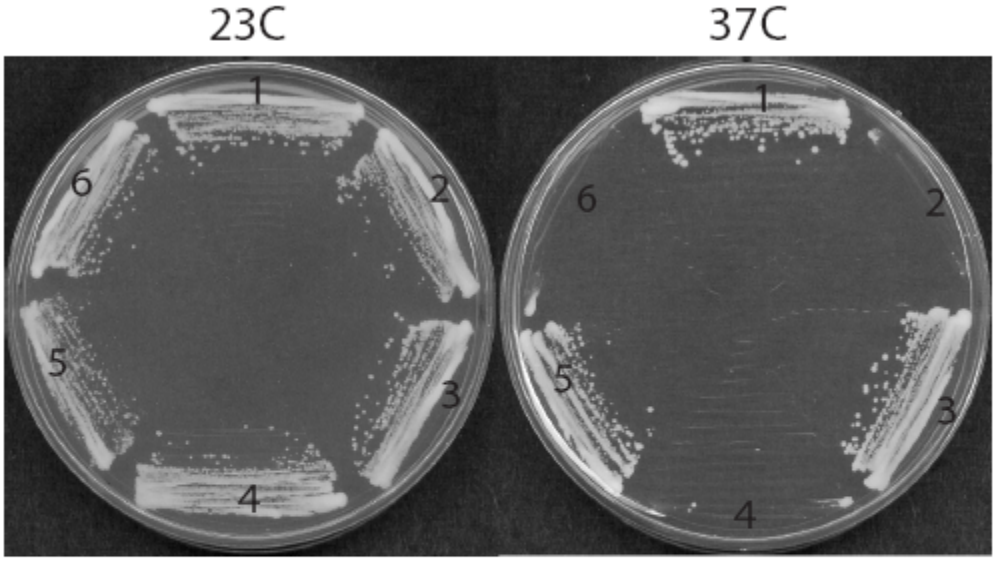
allele is dominant, then the diploid strain will form colonies at the temperature non-permissive for *prp38-1*. In contrast, recessive suppressor mutations will be masked in the heterozygous state and not support growth. All eleven extragenic suppressor strains failed to grow at 37^{o}C indicating that each resulted from a recessive suppressor allele.

Since the suppressor alleles are recessive, candidate suppressor genes could be identified by scoring for reacquisition of the ts phenotype after transformation with a wild type gene copy. I used two of the “best” suppressors (based on the colony size of the suppressed strain) for the initial gene isolation.

I transformed the suppressor strain M3 with a yeast genomic DNA library prepared from wild type yeast (Figure 3.1). Transformants from a single-copy (centromeric) plasmid library were selected by complementation of a plasmid-linked nutritional marker (*URA3*) at the permissive temperature (23°C) and then screened by replica plating on nutrient rich medium at 37°C. In total, approximately 30,000 plasmid transformants were screened. Two plasmids were recovered from yeast that, by the replica plate assay, reacquired temperature sensitivity.

While the yeast used for plasmid recovery clearly showed the ts phenotype, it was possible that this was due to a spontaneous mutagenic event in the transformant rather than the desired plasmid-linked event. In order to address this, I recovered plasmids from yeast, amplified these in *E.coli* and re-

FIG. 3.1 *spp382-1* is a recessive, extragenic suppressor of the spliceosome maturation mutant, *prp38-1.* Growth of wild type yeast (1, 5), an otherwise isogenic *prp38-1* (ts192) mutant (2, 6), and the suppressed mutant (3, 4) on rich media after 3 days at the permissive temperature (23 °C) or two days at the restrictive temperature (37 °C). In streaks 4-6, a centromeric yeast plasmid with the wild type *SPP382* gene (*pSPP382*) is co-expressed.



1. *PRP38, SPP382*
2. *prp38-1, SPP382*
3. *prp38-1, spp382-1*
4. *prp38-1, spp382-1, pSPP382*
5. *PRP38, SPP382, pSPP382*
6. *prp38-1, SPP382, pSPP382*

transformed the each of purified DNA back into the original suppressor strain (M3). Only one of the two recovered plasmids resulted in all the secondary transformants showing the reacquisition of the temperature sensitive phenotype, demonstrating a plasmid-dependent event. I noted that transformation of this same plasmid into a wild type strain did not change its growth characteristics at either 23 °C or 37 °C (Figure 3.1). Confirmatory results for plasmid linkage of the ts phenotype were obtained by counterselecting for plasmid loss from the secondary transformants by growth in the presence of the anti-metabolite, 5-fluroorotic acid (Boeke, LaCroute et al. 1984). As predicted based on linkage, plasmid loss resulted in colonies which were no longer temperature sensitive. Thus, the identified plasmid (but not a control plasmid assayed in parallel) was sufficient to block the recessive suppressor phenotype and thus contained a yeast gene that might encode the wild type allele of the suppressor locus.

The candidate plasmid was sequenced to find out which chromosomal region it contained. The DNA fragment had two ORFs (*ATG17* and an unknown open reading frame, *YLR424w*) from chromosome 12. *ATG17* codes for a non-essential scaffold protein that plays a role in pre-autophagosomal structure organization (Kabeya, Kamada et al. 2005) and seemed to be unrelated to splicing. On the other hand, we and others had observed the *YLR424w* gene product in splicing complexes (Gavin, Bosche et al. 2002); (Wang, Hobbs et al. 2003) making this the likely candidate. This gene was sub-cloned using PCR into a centromeric plasmid. Transformation of this clone into the naïve M3 strain resulted in reacquisition of temperature sensitivity (Figure 3.1). Confirmation that

this was the suppressor-defined gene was made by linkage analysis which established that the cloned DNA was inseparable from the original suppressor locus (see Materials and Methods). I also sub-cloned *ATG17* from the original library plasmid using restriction enzymes and transformed into the original suppressor strain. No loss of suppression was observed with this gene, establishing *YLR424w* and not *ATG17* as the suppressor gene.

Reduced Spp382p activity suppresses the prp38-1 defect.

SPP382 was reported as essential in the systematic analysis of yeast gene function study (Giaever, Chu et al. 2002) but Spp382p's role, if any, in cellular splicing was undetermined. To investigate this, I tested a series of site-directed mutations in residues conserved in the yeast, human, zebra fish, fruit fly and nematode homologs (Altschul, Madden et al. 1997) for biological activity (Figure 3.3 A). The plasmid borne *spp382* mutants were scored for complementation of the null *spp382::KAN* allele after plasmid shuffle removal of a *URA3*-linked wild type copy of this gene (Figure 3.3 A). *SPP382* encodes a conserved 708 amino acid protein that contains a G-patch motif common to a number of RNA binding proteins (Figure 3.2 B) (Aravind and Koonin 1999; Silverman, Maeda et al. 2004; Tsai, Fu et al. 2005; Herrmann, Kais et al. 2007). Changes in the G- patch domain either prove lethal (*spp382-9* or *spp382-10*) or impair growth (*spp382-2* and *spp382-3*) consistent with this being a critical motif (Fig 3.3 B and Table 3.1). A small deletion within the central domain results in a tight ts mutant (*spp382-5*). Mutants bearing triple alanine substitutions near the

FIG. 3.2. Alignments of the G-Patch and Spp382p protein. A. Alignment of Spp382p homologs showing conservation of the protein across species. The abbreviations used are C.e – *Caenorhabditis elegans*, D.r – *Danio rerio*, H.s – *Homo sapiens,* D.m. *Drosophila melanogaster*, S.c - *Saccharomyces cerevisiae.* **B.** Alignment of G-patch motif with the consensus below. The sequences are named using the notation Protein name _ Species abbreviation_Gene Bank GI (Gene identification number). Abbreviations used Sc - *Saccharomyces cerevisiae*, Hs - *Homo sapiens*, Sp -*Schizosacchromyces pombe*, Ca -*Candida albicans*, At - *Arabidopsis thaliana*.

A

```
D.r.   1 MSMSHLYGRRGDEEEEDGVEIEKFEVSEWDLANEFNPDRRRYRQTKEEATYGIWAEQDSD
60
H.s.   1 MSLSHLYRDGEGRIDDDDDERENFEITDWDLQNEFNPNRQRHWQTKEEATYGVWAERDSD
60
D.m.   1 ---------------MSDNDYERFEITDYDLDNEFNINRPRGRQSRHQQIYGIWADDSEE
45
C.e.   1 --------------MEDDDGRESFEINDMDLEYAMNPGGRRRFQNKDQATYGVFAPDSDD
46
S.c.   1 ----------------------------MEDSDSNTDKKFFFKKRRIDSYNYSDEEDNN
31
                                        * .     :.:    *.     ..:

D.r.   61 DERPSFGGKR-------AKDYSTPVSFVSAGLRKTAAEEKAEREGSDDS-------DAEE
106
H.s.   61 DERPSFGGKR-------ARDYSAPVNFISAGLKKGAAEEAELEDSDDEE-------KPVK
106
D.m.   46 ESGGEGGTKRRGRAARKPKDYTMPVNFVAGGIQQAGKKKKKALQADDEKGSQKEGAEADQ
105
C.e.   47 DDDEQGTSRG---PYKKRSKISAPMSFVSGGIQQGNKIDKDDPASLNLN-------LGGE
96
S.c.   32 SSMNS------------DMTYTNDALKTSSGNAPTISKLTK------------------
60
          ..  .               :      :.*

D.r.   107 APPPPRAAAPKKLQTGGSFKTSQR-FAGGIR------------------TGQDLGNWEK
146
H.s.   107 QDDFPKDFGPRKLKTGGNFKPSQKGFAGGTK------------------SFMDFGSWER
147
D.m.   106 GEESDDSAASGRPAFGQNDPGSSNSSSEEERPTLSRKQPSTTFQHRSHIASERNVGAWEQ
165
C.e.    97 KKPKEDDEGSIQIDFDKRTKKAPKQNGAQVFAGMRSSANHG-------AADINQFGSWMR
149
S.c.       ------------------------------------------------------------


D.r.   147 HTRGIG--QKLLQKMGYVPGKGLGKNAQGIVNPIEAKLRKGKGAVGAYGSERTQQS---L
201
H.s.   148 HTKGIG--QKLLQKMGYVPGRGLGKNAQGIINPIEAKQRKGKGAVGAYGSERTTQS---M
202
D.m.   166 HTRGIG--AKLLLQMGYEPGKGLGKDLQGISHPVQAHVRKGRGAIGAYGPETAASIGGKT
223
C.e.   150 GDGNSNKIMKMMQAMGYKPGEGLGAQGQGIVEPVQAQLRKGRGAVGAYGKESTATGPKFG
209
S.c.    61 -TYGIG--AKLLSSMGYVAGKGLGKDGSGITTPIETQSRPMHNAGLGMFSNTNSSNYHSE
117
             . .   *::  *** .*.*** : .**  *:::: *  :.*  .   :

D.r.   202 QDFPVVDSEEEEEEEFQKELGQWRKEPGT-------AKKKPKYSYRTVDDLKAHGTRANM
254
H.s.   203 QDFPVVDSEEEAEEEFQKELSQWRKDPSG-------SKKKPKYSYKTVEELKAKG-RISK
254
D.m.   224 NKSIKVDEDVREAKEFKDQLNKWRKGSAGGAE--PMERQGKRYYYKSVEEVIAKGHTSGH
281
C.e.   210 ESAADAQKRMAQEGTSSRPTNDDQEKSGLKIKGSWKKSQTVKTKYRTIEDVMEEGMSASR
269
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S.c.   118 NEDYLSSEDEVVEGIEQVKFNKTSTEVLG-------EALLNDSGDMTIVRTLRELRLAGV
170
           :.    ..        .   ..                        ::     .    .

D.r.   255 SMSRPA-GELAQVKVIDMTGREQKVYNSYSHMSQKHSVP------EEAPLSVSTREQKSS
307
H.s.   255 KLTAPQ-KELSQVKVIDMTGREQKVYYSYSQISHKHNVPDDGLPLQSQQLPQSGKEAKAP
313
D.m.   282 LLSEKLSKKLGNVRVIDMTGPEKRVLSGYHALGQAKITPEETLYDTEATEKGS---APAC
338
C.e.   270 PASHQQSQQYSNIKVIDMTGKQQKIYSGYDSFSMKTRSEYDTVDDEERT----------
318
S.c.   171 QLPESILKELDPLNAVPKPKKDVVVEILQELLGIEKSLEAIRQR-----------TSPL
218
             .     :   :..:  .  :  :      :.

D.r.   308 GFALPELEHNLKLLIELTEQDILQSARLLQHEKDTVVTLTHESDALQVRLAEEEETLGRL
367
H.s.   314 GFALPELEHNLQLLIDLTEQEIIQNDRQLQYERDMVVNLFHELEKMTEVLDHEERVISNL
373
D.m.   339 VFAMPELTHNLQLLVSQCEQQIIAIDNQERECSSQQAALESEHRKLEEIVQLERNHIRTL
398
C.e.   319 VFDVPELIHNLNLLVDLTEEGIRRSNQQLISLKDQTTALEYDLQQVQKSLGTEEQEAQHI
378
S.c.   219 EVQVKEYYGQERLLSELEVTLRDESKHVSLYDKIGAILKLSDDELIDRLTSCLLRKELLI
278
            . : *   : .** .          .             :   :        .    :

D.r.   368 EQVMSLVERFEAGDKEGDP-ALSLQECAKIFEQLQTEFYQEYKTMGLGDLAVSVVHPLLK
426
H.s.   374 SKVLEMVEECERRMQPDCSNPLTLDECARIFETLQDKYYEEYRMSDRVDLAVAIVYPLMK
433
D.m.   399 EESLERVERLIDNPD------LSLPQAERLFRELLVDYAAEFHEFGLADLAAGVIAPLLK
452
C.e.   379 KDVYELIDGFSSNRSP------SMEECQELFRRLRSEFPHEYELYSLETVAIPTVLPLIQ
432
S.c.   279 EFDLDHLEKPNDILD-----------ELTQIIELLAYRMDTTSKFLNRTQTTIFKVIYP
326
           .   . ::      .                      :  :            .  :


D.r.   427 EKLRNWDPLKDCSDGLEEVGQWRAILESTL------SLHSGPDTTNMDPYHRLIWEVWVP
480
H.s.   434 EYFKEWDPLKDCTYGTEIISKWKSLLENDQ------LLSHGGQDLSADAFHRLIWEVWMP
487
D.m.   453 RELVQWQPLENPTEPLPLIKKWRGMLQQGD------AAEQQPR-NVFDPYSSLIWAGVMP
505
C.e.   433 KYFVAWKPLEDKNYGCELISTWRDILDDSKNGRKMTFGHNKTKGDEIRAYDRIIWEGILP
492
S.c.   327 KLKKFWEGFDMTKSKIDSAITLLLDFQQVLS-------------FIGCKEHIMEEFVYP
372
           .    *. :.  .           ::.                       ::     *
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D.r.   481 VMRTCVSQWQP-RNVGPMVD--CVECWAPVLPLWILDHVLEQLIFPRLQREVDNWNPLTD
537
H.s.   488 FVRNIVTQWQP-RNCDPMVD--FLDSWVHIIPVWILDNILDQLIFPKLQKEVENWNPLTD
544
D.m.   506 SFRSSAAAWQP-KEHPPMAS--LLDAWAPLLPSWVLDSVLEQLVLPRLVAGVQEWDPLTD
562
C.e.   493 SIRRACLQWDPSTQMHEMIE--LVEQWIPLLSAWITENILEQLVVPKIAERVNQWDPMTD
550
S.c.   373 KLLQELDNWELHDEVDHVSPRIWVLDFMVLIDDKIKDTIVDKIEAKFFAYCKNWYHRESF
432
                .      *:   :   :     :  :  ::   : : :::::    :    : :.  :

D.r.   538 TVPIHSWIHPWLPLMQTRLEP-LYAPIRSKLAHALQRWHPSDSSARLILQPWRDVFTPGA
596
H.s.   545 TVPIHSWIHPWLPLMQARLEP-LYSPIRSKLSSALQKWHPSDSSAKLILQPWKDVFTPGS
603
D.m.   563 TVPIDSWVLPWHAILGSKLEEAVYPQIRSKLGIALRAWSPHDRSARAMLTPWQKAFPEEE
622
C.e.   551 EIPIHEWLVPWLVLLGDRIQT-VMPPIRQKLSKALKLWDPMDRSALETLRPWQNVWSAAT
609
S.c.   433 CITNSDIIFIKELICERRYYKILCKEFLPKFLDELWERHNDPIYELEDWKEKQEWKEKDS
492
                :.  . :     :   :    :   :  *:   *                 :.

D.r.   597 WEAFMVKNIVPKLALCLGELVVNP-HQQLLDPFNWVMDWECMLSVSSMVGLLDKNFFPKW
655
H.s.   604 WEAFMVKNIVPKLGMCLGELVINP-HQQHMDAFYWVIDWEGMISVSSLVGLLEKHFFPKW
662
D.m.   623 MQEFLQRYIVPKLQATLGELIIIP-MHQDLELWQQVWEWHELIDPMYMAQLLDRHFFPRW
681
C.e.   610 FSAFIAQNIVPKLGVALDTMELNPTMNPEYPEWTACMEWLEFTHPDAIANIVTKYFFPRF
669
S.c.   493 GFFYFMKKLRSYTHYFHPKQYELMMRGTFNNINKILYQWHLYS---TVEDLHKSKWWLNW
549
              :: : : .                           :*        :  :    :: .:

D.r.   656 LQVLCSWLSN-NPNYEEITKWYLGWKGLLSENLLSHPLVKEKLNEALDIMNRAVASGLGG
714
H.s.   663 LQVLCSWLSN-SPNYEEITKWYLGWKSMFSDQVLAHPSVKDKFNEALDIMNRAVSSNVGA
721
D.m.   682 MQVLVVWLNQ-SPDYAEISRWYTGWKSMLSEPLLREPSVKEHLRRALEIMHRASDTLLQP
740
C.e.   670 YNCLCLWLDSPGVDYNEVKRWYGSWKARIPQVLVNYPTVNENLRRSMIAIGRSLQGEKVG
729
S.c.   550 LMNTVFEHSLPTEIELSEIRKSYNIFAMSHRYHLDKSTLDEDFDLRQGLRNLMETQVIDD
609
                       .                    .  :   .  .     :  . :.:.:

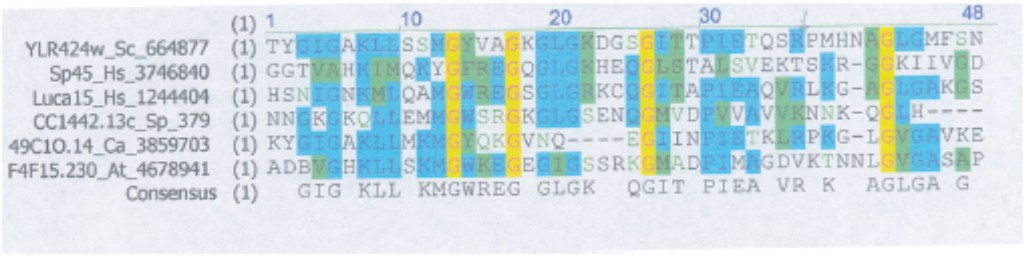
D.r.   715 YMQPGARENIAYLIQTERRKDFQCELQPERREVDNSATRPPGMAAVPASVPTNFKDLVQA
774
H.s.   722 YMQPGARENIAYLTHTERRKDFQYEAMQERREAENMAQR--GIGVAASSVPMNFKDLIET
779
D.m.   741 TVTPTPPP----------------------PVPPAPVIMMDLIHPPAQLEFKELVSQ
775
C.e.   730 GLQATPIAPMAP----------------------PPPMAPHFTQAAPVQKLSLKEIIEY
766
```

```
S.c.   610 ISQSEQEP-------------------------TYTVQNIPLGKVSSSFKDVVEDYCLE
643
              .                               .     :    .     .::


D.r.   775 KAEENGIVFMPLVAKRHMGKQLFTFGRIVIYIERGVVFVQGE--KTWVPTSLQSLIDMAK
832
H.s.   780 KAEEHNIVFMPVIGKRHEGKQLYTFGRIVIYIDRGVVFVQGE--KTWVPTSLQSLIDMAK
837
D.m.   776 QCADLGIIFAPLPGRREMGKQIYRVGKLFCYIDRHVCMVSDGSFSNWKPVSLNHLLERSQ
835
C.e.   767 TAGKNGFTYHPQKDRYKDGRQVFWFGALSIYLDSEMVYVMDPIEFVWRPSGLNELIQMAQ
826
S.c.   644 KGYLISKIPNRYTQLPYGRDQDCIVPLFEIRNGKKKMEVALKHDILWVEDSSGTFKPIYL
703
               .              *   .  :          *       *   .   :

D.r.       -----
H.s.       -----
D.m.   836 TGIL- 839
C.e.   827 GAQG- 830
S.c.   704 WALDL 708
```

B

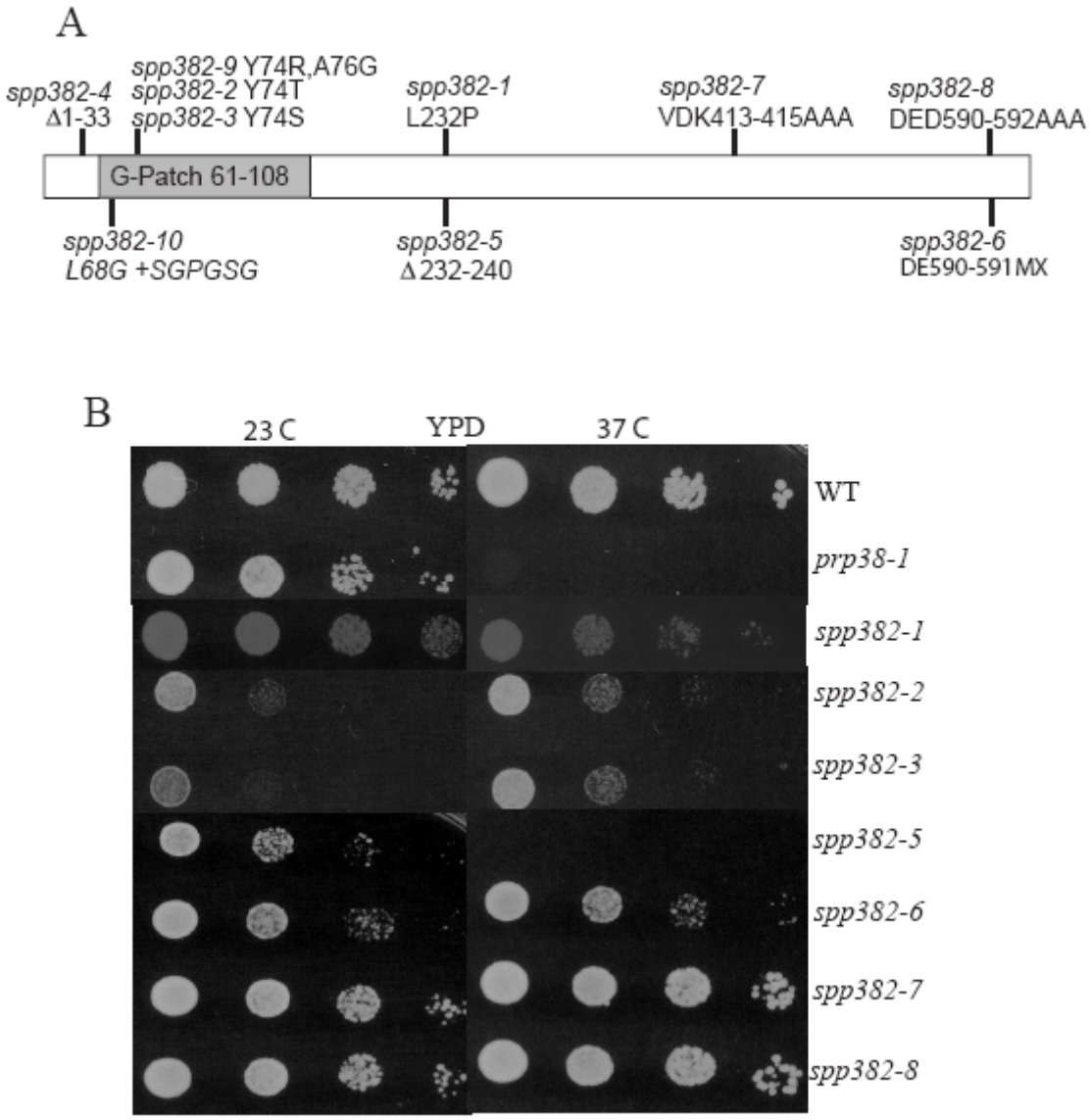

(1) 1 10 20 30 48
YLR424w_Sc_664877 (1) TYGIGAKLLSSMGYVAGKGLGKDGSGITTPIETQSRPMHNAGLGMFSN
Sp45_Hs_3746840 (1) GGTVAHKIMQKYGFREGQGLGKHEQGLSTALSVEKTSKR-GGKIIVGD
Luca15_Hs_1244404 (1) HSNIGNKMLQAMGWREGSGLGRKCQGITAPIEAQVRLKG-AGLGAKGS
CC1442.13c_Sp_379 (1) NNGKGKQLLEMMGWSRGKGLGSENQGMVDPVVAVVKNNK-QGLH----
49C10.14_Ca_3859703 (1) KYGIGAKLLMKMGYQKGVNQ----EGIINPIETKLPPKG-LGVGAVKE
F4F15.230_At_4678941 (1) ADBVGHKLLSKMGWKEGEGIGSSRKGMADPIMKGDVKTNNLGVGASAP
Consensus (1) GIG KLL KMGWREG GLGK QGIT PIEA VR K AGLGA G

C-terminus (*spp382-7* or *spp382-8*) show wild type growth whereas a nearby frameshift mutation results in slowed growth (*spp382-6*). A *GAL1* driven N terminal deletion (*spp382-4*) that shifts the translational start to codon 34, supports efficient cell growth on galactose based medium (Fig 3.3 B and 3.3 C).

I noted that the *spp382-1* mutant grows less well when expressed from this plasmid than when expressed from its normal chromosomal locus. I speculated that this might be due to a too-short 3'UTR in the plasmid construct. To test this, I re-created the construct with a longer 3' UTR (original clone was 2527 base pairs (bp) ending at the stop codon while the 3' UTR clone is 2677 bp with additional 150 bp downstream of the stop codon) and this strain grows as well as the chromosomal integrant (Fig 3.3 C). This observation suggests that the too-short 3' UTR exacerbates the *spp382-1* growth defect and may contribute to the weaker growth of the other *spp382* mutants. However it should be noted that the short 3'UTR by itself is not particularly inhibitory, since the *spp382-7* and *spp382-8* clones grow as well as wild type.

Next the newly created *spp382* mutants were tested for the ability to suppress the *prp38-1* defect. The plasmid based *spp382* mutants were transformed into a *prp38-1* strain with a chromosomal deletion of the *SPP382* locus (i.e., *spp382::KAN*) and a *URA3*-linked wild type allele of *SPP382.* The *spp382* mutant alleles were checked for suppression by growing the strain at 37 °C after removal of the *URA3*-linked *SPP382* by plasmid shuffle. Suppressor alleles were found distributed throughout the coding sequence (i.e., *spp382-1,*

FIG. 3.3 Spp382p is essential for cell viability. A. PCR-based mutagenesis was targeted to conserved regions of Spp382p (based on UniProtKB entries: Q06411, Q9UBB9, Q6DI35, Q9NHN7, and Q17784). Panel A shows the amino acid changes and numbered coordinates on the 708 amino acid protein. **B.** Growth assays performed in yeast with the indicated *spp382* allele at 23 °C and at 37 °C. **C.** Growth assay showing the effect of shortened 3' UTR. The growth of a plasmid borne *spp382-1* with the short 3' UTR (*spp382::KAN, p-spp382-1*), a plasmid borne *spp382-1* with elongated 3'UTR (*spp382::KAN, p-spp382-1 + 3'UTR*), and that of a chromosomal *spp382-1* strains at 23 °C and 37 C compared to wild type and *prp38-1* mutant. It should be noted that the figure B and C are a composite of multiple plate cultures incubated at the indicated temperatures for identical periods of time.


A
spp382-9 Y74R,A76G
spp382-4 spp382-2 Y74T
Δ1-33 spp382-3 Y74S
spp382-1
L232P
spp382-7
VDK413-415AAA
spp382-8
DED590-592AAA
G-Patch 61-108
spp382-10
L68G +SGPGSG
spp382-5
Δ232-240
spp382-6
DE590-591MX
B
23 C
YPD
37 C
WT
prp38-1
spp382-1
spp382-2
spp382-3
spp382-5
spp382-6
spp382-7
spp382-8

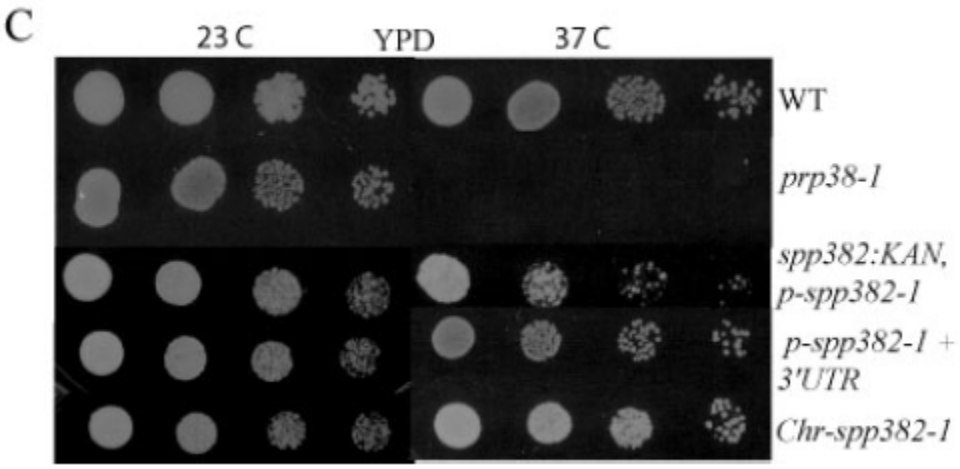

C
23 C
YPD
37 C
WT
prp38-1
spp382:KAN,
p-spp382-1
p-spp382-1 +
3'UTR
Chr-spp382-1

Table 3.1 Suppression by titration of an essential splicing activity. Visible colonies from an otherwise inviable *prp38-1*mutant were indicative of suppression (Yes) while no growth indicates no suppression (No). NA means not applicable. * indicates that this allele was expressed with the *GAL1* promoter, all other alleles were expressed with the *SPP382* promoter. ** indicates the strain is lethal.

Allele	*prp38-1* suppression
SPP382	NA
prp38-1	NA
spp382-1	Yes
spp382-2	No
spp382-3	No
*spp382-4**	Yes
spp382-5	Synthetic Lethal
spp382-6	Yes
spp382-7	No
spp382-8	Yes
*spp382-9***	NA
*spp382-10***	NA

spp382 -4, spp382 -6 and spp382-8). The tightly ts allele, *spp382-5*, is lethal in the *prp38-1* background. None of the mutations were found to suppress a lethal *prp38::KAN* null allele and similarly the *prp38-1* mutation does not rescue the lethality of the *spp382-9* or *spp382-10* mutations (Table 3.1).

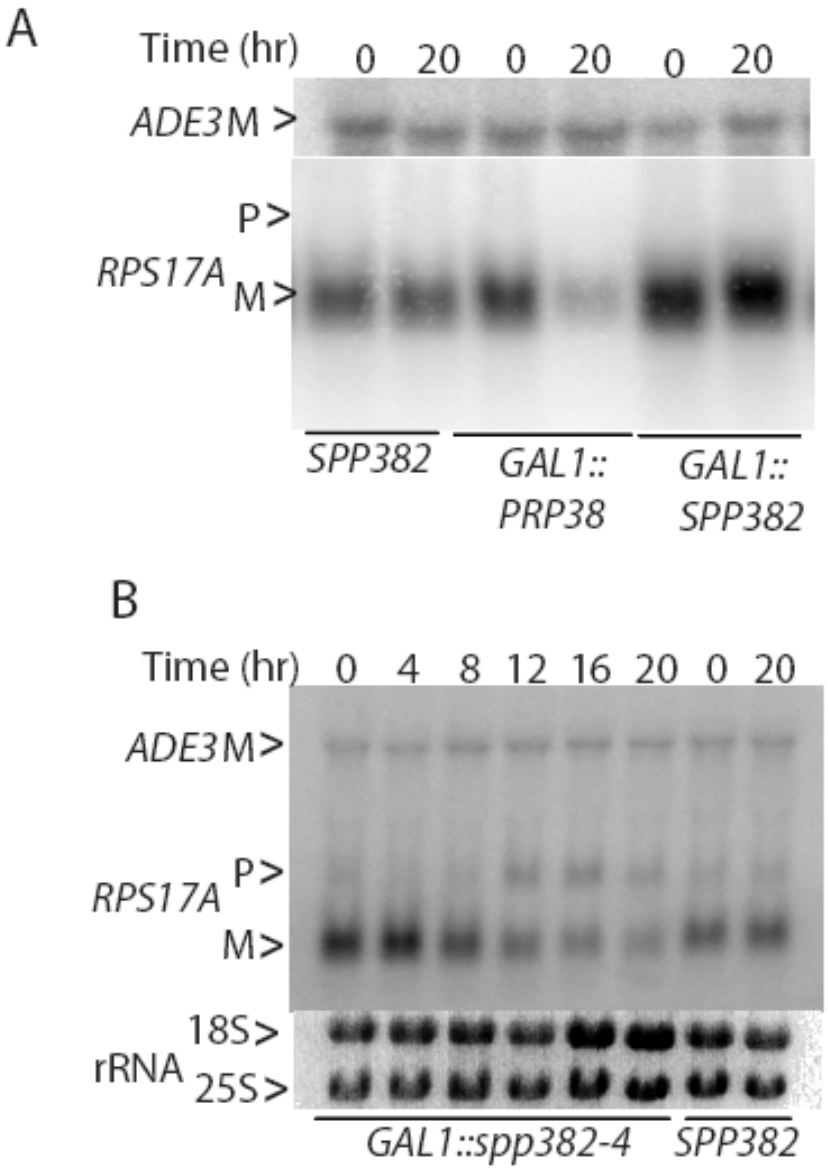
Spp382p is required for splicing and for normal intron metabolism.

Incomplete repression of transcription prevented the use of *GAL1::SPP382* to assess the cellular role of Spp382p in splicing (Fig 3.4A). But the metabolic depletion can be achieved using the functional *GAL1::spp382-4* allele which shows time dependant inhibition of splicing and growth (Fig 3.4 B and data not shown). Under this condition, no change is observed in the intronless *ADE3* transcript or the mature rRNA (Fig 3.4 B).

The level of splicing inhibition observed with the *spp382* point mutants was shown to parallel the growth impairment (Fig 3.5). Mutants with strong growth defects (*spp382-1, spp382-2, spp382-3*, *spp382-5*, and *spp382-6*) show significant splicing inhibition, especially at higher (37 °C) temperature. Mutants that support robust growth show little splicing inhibition (e.g., *spp382-7*, *spp382-8).*

Mutations in some splicing factor genes, especially those splicing factors involved in spliceosome disassembly, accumulate excess levels of excised intron (Arenas and Abelson 1997); (Martin, Schneider et al. 2002). It is believed that the residual bound splicing factors inhibit the turnover pathway. I decided to probe whether excess intron accumulates in the *spp382* mutants by probing my

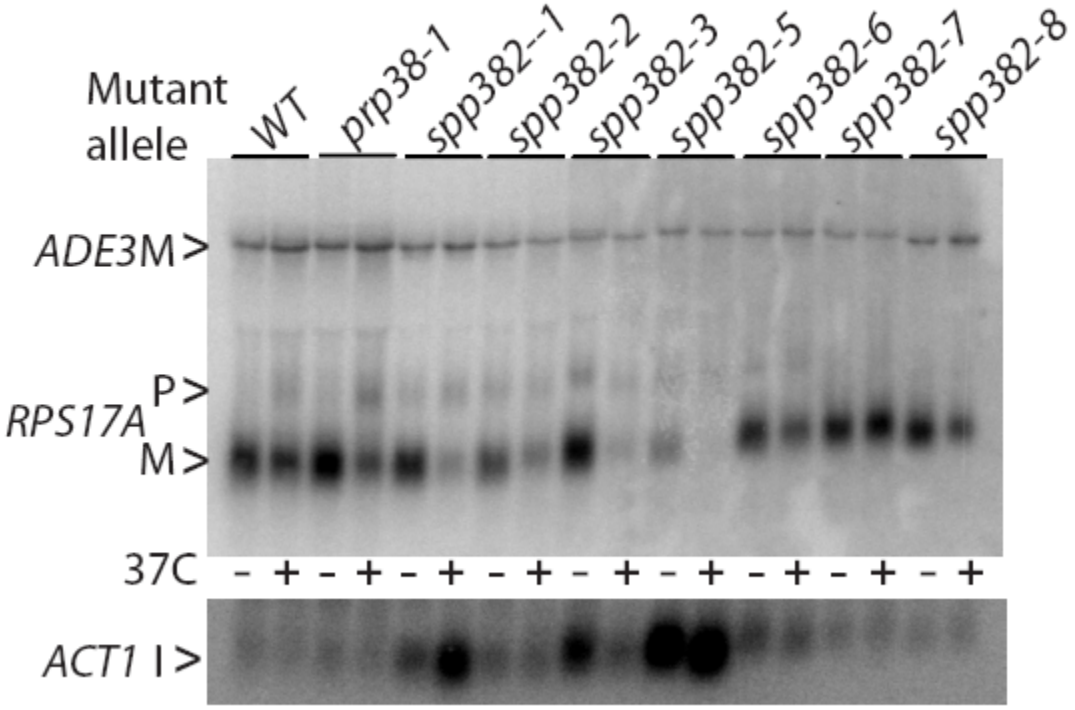
FIG 3.4. Spp382p is required for splicing. Northern analysis of splicing inhibition with yeast that express the nutritionally regulated *GAL1::SPP382* (Panel **A**) or *GAL1::spp382-4* gene (Panel **B**) (T=0) and 4 to 20 hours after transcriptional repression by glucose. Yeast that express the wild type allele (*SPP382*) and were assayed in parallel after growth in galactose (T=0) or glucose (T-=20). The positions of the intronless *ADE3* mRNA and the *RPS17A* pre-mRNA and mRNA and the mature rRNA are shown to the left.


A
Time (hr)
0
20
0
20
0
20
ADE3
M >
P>
RPS17A
M>
SPP382
GAL1::
PRP38
GAL1::
SPP382
B
Time (hr)
0
4
8
12
16
20
0
20
ADE3
M>
RPS17A
P>
M>
18S>
rRNA
25S>
GAL1::spp382-4
SPP382

northern blot with the *ACT1* intron (Fig 3.5, lower panel). For unknown reasons, *ACT1* is more sensitive in this assay than *RPS17A*. I quantified the intron accumulation using ImageQuant software. The intron accumulation is greatest with the central domain mutants, *spp382-1*(9 fold more at 37 C) and *spp382-5* (15 times more than control at RT and 23 fold more at 37 °C) and with one of the G-patch mutants, *spp382-3* (9 times increased at RT) when compared with the wild type*. spp382-6* only accumulates about 2 times more intron, when compared with the control. There seems to be no simple correlation between intron accumulation and suppression. For instance, while *spp382-1* accumulates excess intron and suppresses *prp38-1*, *spp382-3* and *spp382-5* accumulate much more excised intron but do not suppress *prp38-1. spp382-6* and *spp382-8* suppress *prp38-1* but accumulate little excess intron. While excess intron accumulation presumably relates to splicing impairment, I note that even this is not a simple relationship. For instance, for *spp382-3* intron accumulation is more evident at 23 °C when there is little or no splicing impairment. However, at 37 °C where splicing inhibition is seen, intron accumulation is not observed.

Presumably, the *spp382-1* suppressed *prp38-1* strain grows better because splicing efficiency is improved. To address this, I compared splicing in the suppressed strain to the wild type and the mutant *prp38-1* parent. Although the mRNA to pre-mRNA ratio (an established measure of splicing efficiency (Rymond, Pikielny et al. 1990) reproducibly increases, the enhancement in splicing is very subtle when assayed with either *RPS17A* or *RPL28.* Based on this, it seems that neither ribosomal protein is likely limiting for growth under the

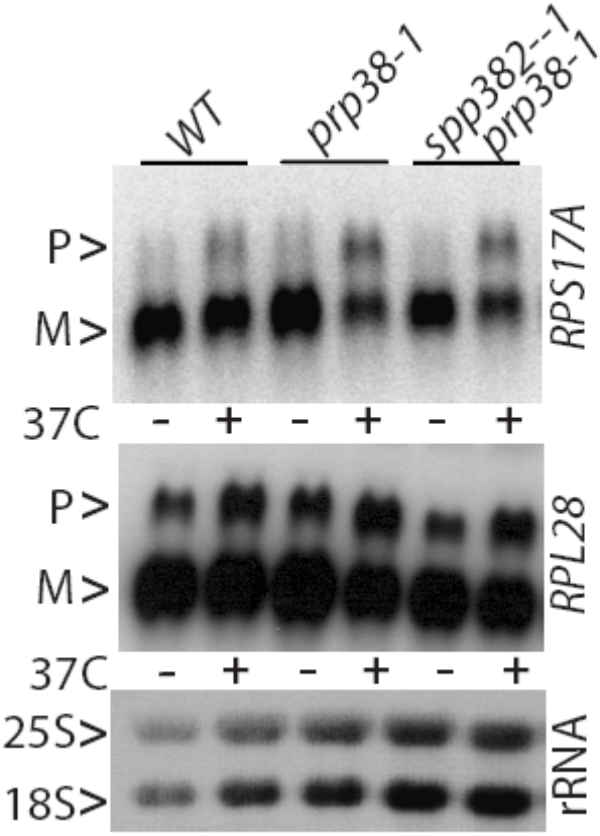
FIG 3.5. Spp382p is required for normal intron metabolism. Northern analysis done with wild type yeast, the *prp38-1* mutant, and the non-lethal *spp382* mutants assayed at room temperature (-) or after 2 hours at 37 °C (+). The lower panel shows accumulation of the *ACT1* excised intron RNA.



Intron accumulation in	25 °C	37 °C
WT *	1X	1X
Prp38-1	1X	1X
Spp382-1	3X	9X
Spp382-2	1X	1X
Spp382-3	9X	1X
Spp382-5	15X	15X
Spp382-6	2X	2X
Spp382-7	1X	1X
Spp382-8	1X	1X

* The intron accumulation is compared to that seen occurring normally in wild type (default (1X))

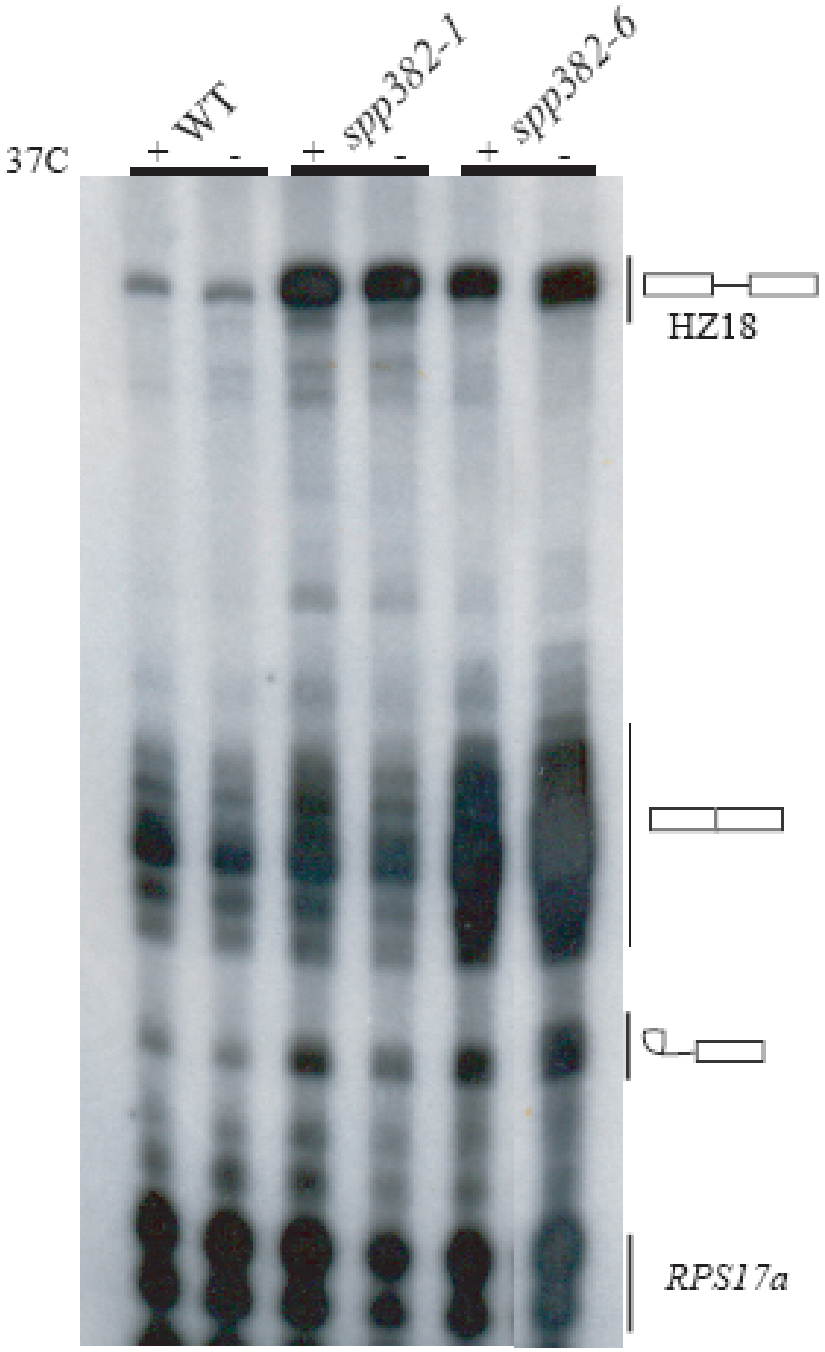
FIG 3.6. Splicing efficiency of the *spp382-1, prp38-1* strain. Splicing of the *prp38-1* mutant and the *prp38-1, spp382-1* double mutant at 23 °C and after 2 hours at 37 °C (+). The 25S and 18S rRNA bands are presented as normalization controls for RNA loading and transfer.


WT
prp38-1
spp382--1 prp38-1
P>
M>
RPS17A
37C - + - + - +
P>
M>
RPL28
37C - + - + - +
25S>
18S>
rRNA

conditions of assay (Fig 3.6), although it is possible that modest decreases to many ribosomal protein genes combine to impair growth.

To find out at which step splicing is blocked after Spp382p inactivation, I performed primer extension analysis. RNA was extracted from strains transformed with a well established reporter gene, HZ18, that contains the intron and flanking exon sequences of the ribosomal protein, *RP51A* (renamed *RPS17A*) fused to lacZ (Teem and Rosbash 1983). Primer extensions were carried out using an oligonucleotide complementary to exon 2, RB1 (Rymond, Pikielny et al. 1990). The primer extension products generated by RB1 include pre-mRNA, lariat intermediate and spliced message (the heterogeneous mRNA population is because of multiple start sites of the *CYC1* promoter). The primer extension analysis shows accumulation of pre-mRNA rather than the lariat intermediate in the mutant *spp382-1* background, even at room temperature (Fig 3.7). Similar results were obtained for *spp382-6* (Fig. 3-7 and for *spp382-2,spp382- 3* and *spp382-5* (data not shown). Thus, while reported dispensable for splicing in vitro (Chan, Kao et al. 2003), Spp382p is essential for first transesterification step in vivo.

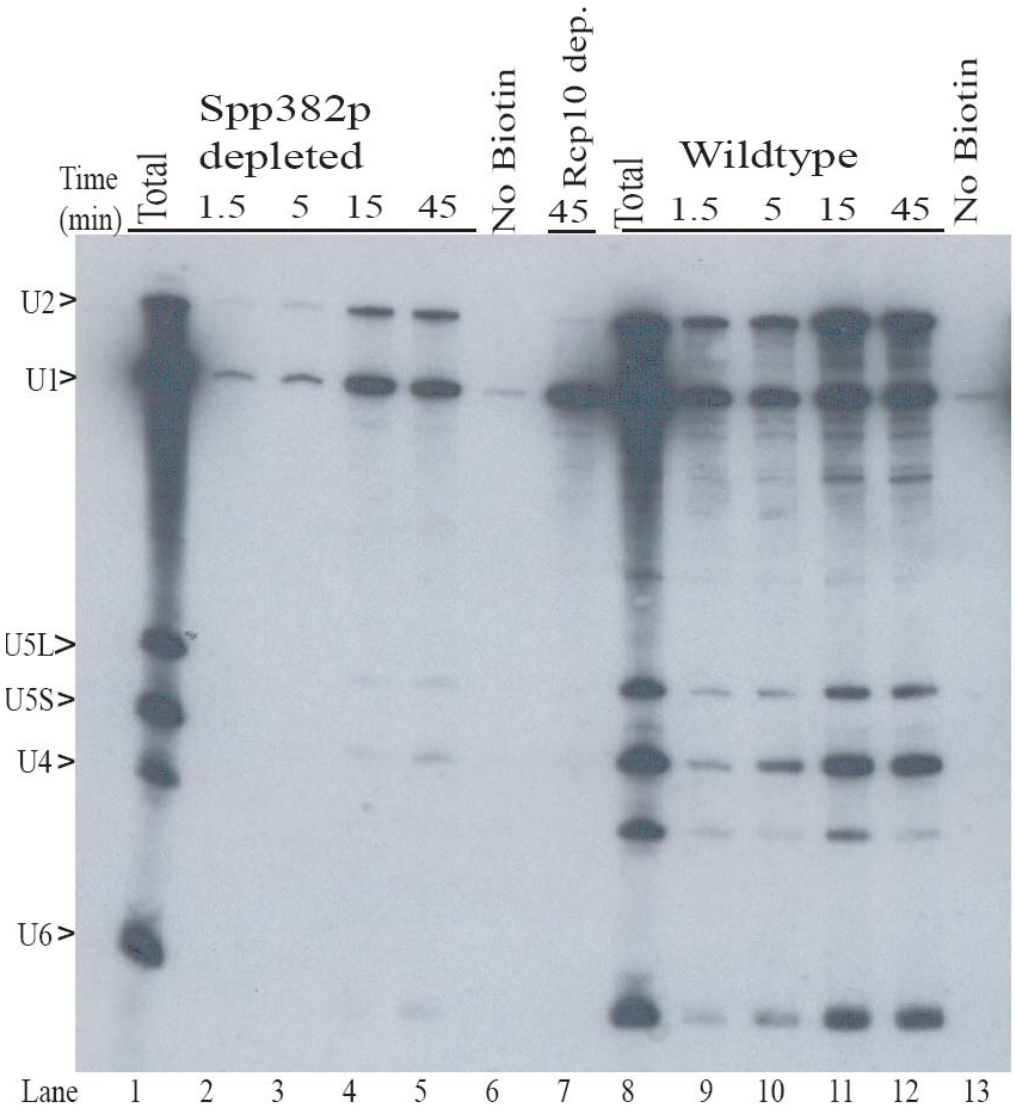
FIG 3.7. Spp382 mutants inhibit the first step of splicing. Primer extension analysis of RNA isolated from *spp382* mutants and a wild type strain using an exon 2 oligonucleotide complimentary to a *RPS17A* reporter. The position of cDNA molecules corresponding to unspliced fusion transcripts (HZ18), spliced fusion message, lariat intermediate and spliced endogenous message is indicated to the right.


37C
+ WT
-
+ spp382-1
-
+ spp382-6
-
HZ18
RPS17a

Extracts from yeast depleted of Spp382p fail to assembly complete spliceosomes.

Spp382p might function directly in splicing or act indirectly, for instance, by stabilizing poorly spliced pre-mRNAs. To investigate the impact of Spp382p depletion on the splicing apparatus, splicing complexes assembled in vitro on biotin-substituted *RPS17A* pre-mRNA were affinity-purified by streptavidin-agarose chromatography and assayed for snRNA content. As seen in Figure 3.8, with the wild type extract, the earliest complexes recovered contain U1 and U2 snRNAs and some U4, U5 and U6 snRNAs. As the assembly progresses, the relative yields of U5 and U6 snRNAs increase. The expected release of U4 snRNA (Xie, Beickman et al. 1998) from the spliceosome can be seen as a time dependant decrease in the recovered U4 snRNA relative to the U6 snRNA (compare lanes 9,12). Note that non-specific association of unmodified (i.e., biotin-free) pre-mRNA or snRNAs with the streptavidin agarose matrix is insignificant (lanes 6 and 13). Metabolic depletion of the essential Rcp10 protein, a protein we recently identified as an essential U2 snRNP SF3b component (Wang, He et al. 2005), blocks assembly after U1 snRNP addition showing spliceosome assembly arrest at the commitment complex stage (lane 7). Metabolicl depletion of Spp382p (see Material and Methods) allows the U2 snRNP to be added (i.e., prespliceosome formation) but assembly arrests at that point. Note that the total snRNA levels remain comparable to wild type (lane 1 and 8) and hence snRNAs are not degraded in the absence of Spp382p. This indicates that, in extracts from cells depleted of Spp382p, impaired U4/U6.U5 tri-

FIG 3.8. Removal of Spp382p blocks splicing complex assembly at the prespliceosome stage. Splicing complexes assembled on biotin substituted *RPS17A* pre-mRNA for the indicated times were affinity purified by streptavidin agarose chromatography and assayed for snRNA content (arrow heads) by northern blot. Total = Total, unselected extract RNA; no biotin = parallel reaction conducted with pre-mRNA lacking biotin.


Spp382p depleted
Wildtype
Time (min)
Total
1.5
5
15
45
No Biotin
45 Rcp10 dep.
Total
1.5
5
15
45
No Biotin
U2
U1
U5L
U5S
U4
U6
Lane
1
2
3
4
5
6
7
8
9
10
11
12
13

snRNP addition limits spliceosome assembly. This block might result from reduced U4/U6.U5 snRNP assembly or from reduced U4/U6.U5 snRNP affinity for the prespliceosome. In either case, genetic depletion of Spp382p reproducibly results in splicing-inactive extracts that fail to assemble splicing complexes beyond the prespliceosome stage.

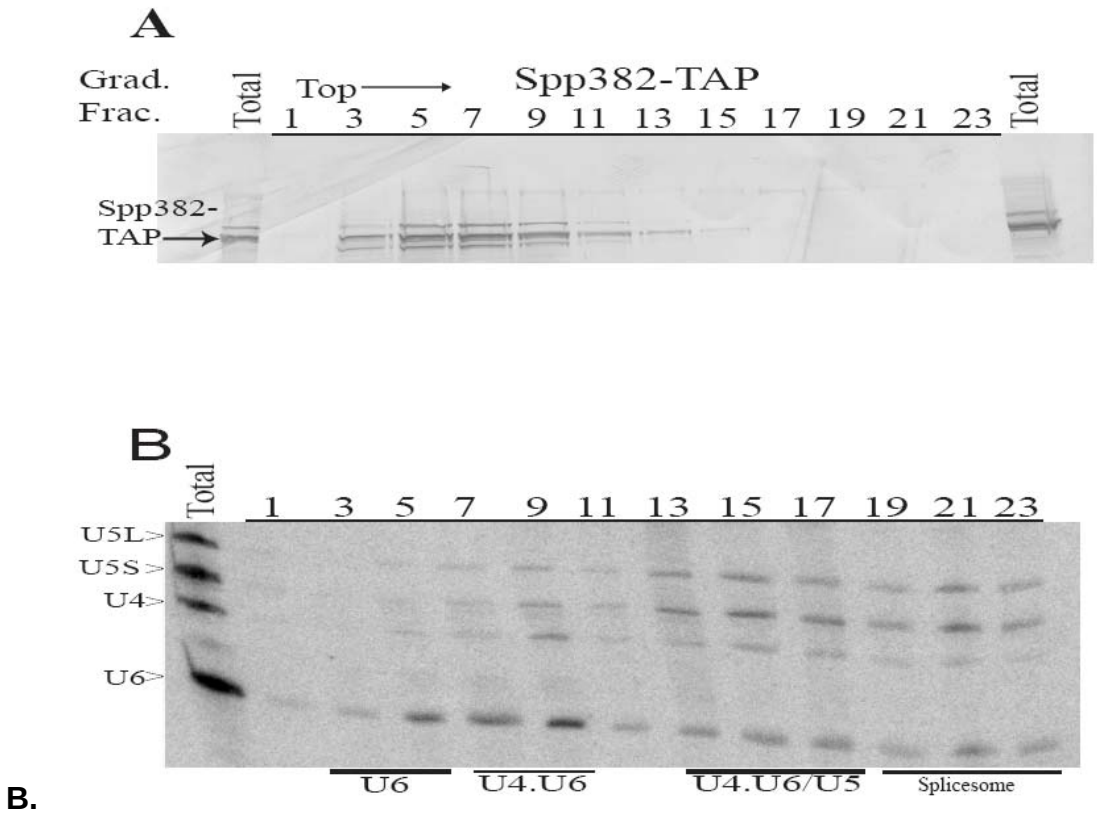
Spp382p is not an integral snRNP protein but its depletion inhibits stable tri-snRNP formation in vivo.

To investigate whether Spp382p is associated with the major spliceosomal snRNP particles, I first looked for evidence of co-sedimentation in a 15-45% glycerol gradient. I checked alternate fractions of the yeast Spp382-TAP extract (an epitope-tagged derivative) for the presence of Spp382-TAP and the splicing associated snRNPs. I note that the *SPP382-TAP* is expressed from its native promoter at its original genetic locus and therefore is likely present at normal levels. 15 µl of extract from the alternate fractions were run on an SDS protein gel and probed with an antibody that binds to the TAP epitope. At the same time, RNA isolated from alternate fractions was resolved on denaturing polyacrylamide gel and hybridized with snRNA-specific probes to see the distribution of snRNPs across the gradient. Most of the Spp382-TAP was found in fractions 3-9 that include the bulk of cellular proteins (mostly fractions 1-7), well separated from the majority of the snRNP complexes. I note that the Spp382-TAP profile does overlap the free U6 snRNP and the U4/U6 di-snRNP (Fig 3.9, Panel A and Panel B). While not shown here, the large U1 and U2 snRNP

FIG 3.9. Spp382p's relationship to the tri-snRNP. A. Extract from cells expressing Spp382-TAP was fractionated on a 15 to 40% glycerol gradient and odd numbered samples assayed by western blot an anti -TAP antibody (lanes 1-23). The lanes marked "Total" show un-fractionated extract.

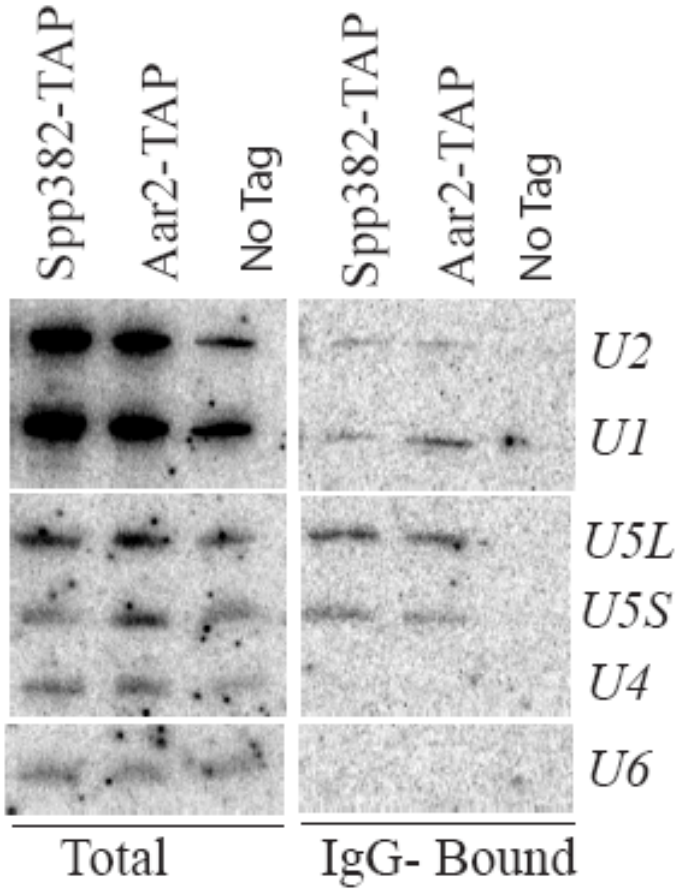
B. Northern analysis of snRNAs present in the glycerol gradient fractions.

C. Northern analysis of snRNAs recovered by immune precipitation with Spp382-TAP, untagged extract, and the U5-specific Aar2-TAP.


A
Grad.
Frac.
Total
Top
Spp382-TAP
1 3 5 7 9 11 13 15 17 19 21 23
Total
Spp382-
TAP
B
Total
1 3 5 7 9 11 13 15 17 19 21 23
U5L
U5S
U4
U6
U6
U4.U6
U4.U6/U5
Splicesome

B.

C


Spp382-TAP
Aar2-TAP
No Tag
Spp382-TAP
Aar2-TAP
No Tag
U2
U1
U5L
U5S
U4
U6
Total
IgG- Bound

particles broadly resolve over the deeper half of the gradient (see Fig. 3.9, below). Based on this, I conclude that under these low stringency salt conditions (150mM NaCl concentration) much of the Spp382p likely exists independent of the major snRNP complex forms driving spliceosome assembly (i.e., U1, U2, U4/U6.U5 snRNPs).

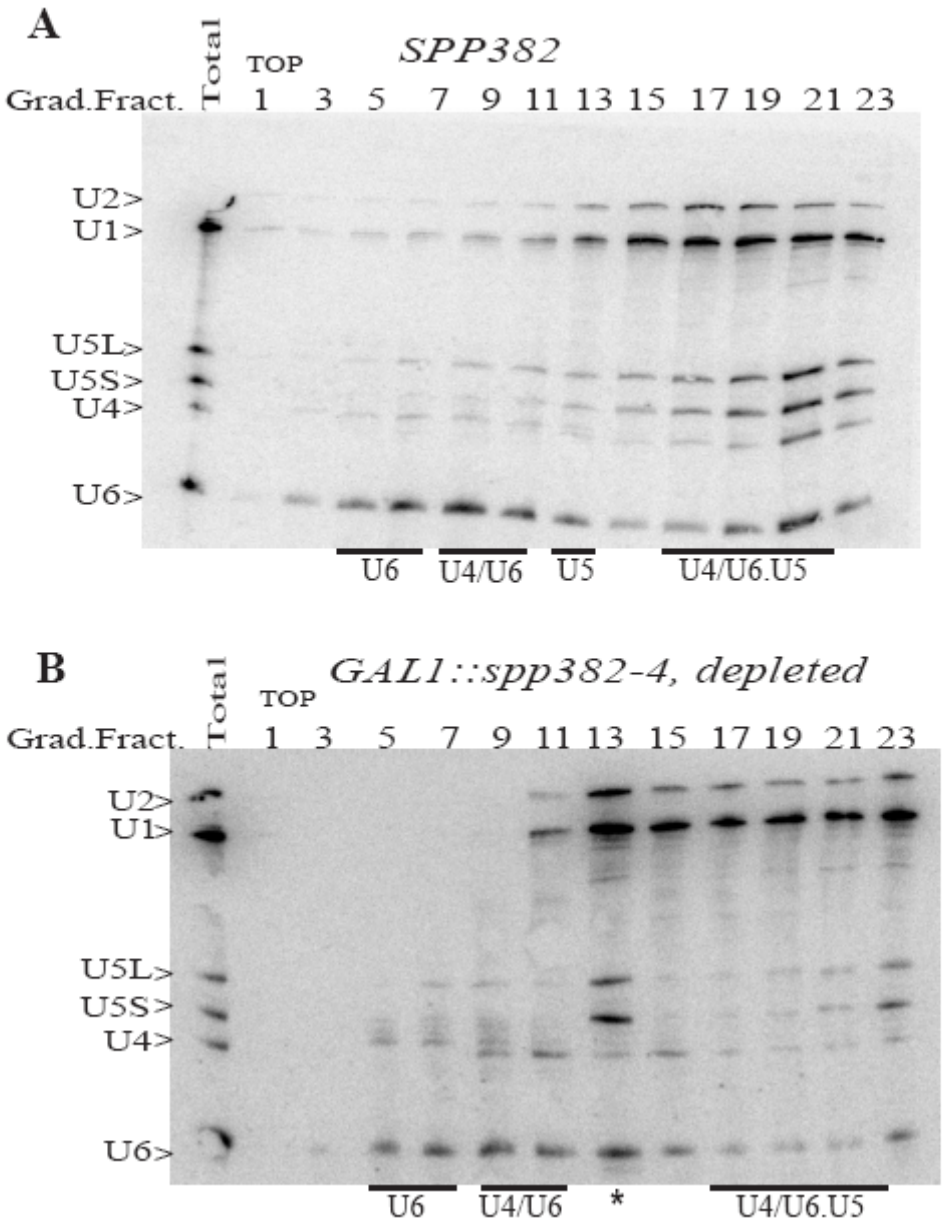
To further investigate whether Spp382p associates with the spliceosomal snRNPs, I next tested whether the U1, U2, U4, U5, or U6 snRNAs co-purify with Spp382-TAP affinity purified on IgG agarose. Northern blot analysis of the recovered RNA (Fig. 3.9 C) shows that Spp382 precipitates mostly the U5 snRNA. While typically 35-50% of the cellular Spp382p could be recovered, no more than 10% (and generally less) of the U5 snRNA was recovered, indicating that Spp382p is unlikely to be a core U5 snRNP protein. This is consistent with previous reports on the yeast U5 snRNP composition (Stevens, Barta et al. 2001). Aar2-TAP, a component of the U5 snRNP and the gene representing the second suppressor identified in my screen (see below), also precipitates U5 snRNA. as well as U1 snRNA as shown previously reported by Fabrizio and Luhrmann (Gottschalk, Kastner et al. 2001). Background binding to the protein A resin is negligible under these conditions (No Tag lane).

I next wanted to address whether Spp382p depletion alters the snRNP composition of a cell. Extracts prepared from yeast with a normal Spp382p content and from a strain metabolically depleted for Spp382p (by glucose repression of *GAL1-spp382-4*) were resolved by glycerol gradient fractionation

and then assayed by northern blot as described above (Fig 3.10) Unfractionated RNA present in the extract is marked as “Total”.

With wild type extract, the free U6 snRNP migrates in the top of the gradient (fractions 3-5, panel A), followed by the U4/U6 di-snRNP complex (lane 7-11, A). The U5 snRNP fractionates across the gradient whereas the tri-snRNP is found near the bottom of the gradient (fractions17-21, A). The final fraction contains, in addition to tri-snRNP particles, endogenous spliceosomes and insoluble aggregates. In contrast to the wild type extract, there is little tri-snRNP present after Spp382p depletion (lane 17-21, B) and the relative amount of U4/U6 complex increases (compare fractions 7-11 in A and B). The asterisk indicates the position of what is likely free U5 snRNP although the co-fractionation of significant amounts of (especially) U6 and U2 may indicate that this is a more complex mixture. Note that the total snRNA levels are comparable in the two extracts (Total, A and B) and hence failure to observe much stable tri-snRNP is not a consequence of snRNA instability. The increase in U4/U6 snRNA levels along with decreased tri-snRNP suggests a defect in the assembly or recycling of snRNP particles for instance, the U2/U5/U6 post splicing complex which might be represented in lane 13.

FIG 3.10. Depletion of Spp382p inhibits stable tri-snRNP formation. Cell extracts of a wild type strain (panel A) or *GAL1::spp382-4* strain (panel B) grown on glucose (to effect metabolic depletion of Spp382p) were layered onto a 15-45% glycerol gradient and the snRNP complexes resolved by centrifugation. RNA was isolated from odd numbered fractions and assayed by northern blot for snRNP composition using snRNA specific probes (lanes 1-23 in panel A and B). Lanes marked "Total" refers to un-fractionated RNA present in the extract. The * in Panel B represents the position of what may be the free U5 snRNP or an aberrant from of the spliceosome (U2, U5, U6).

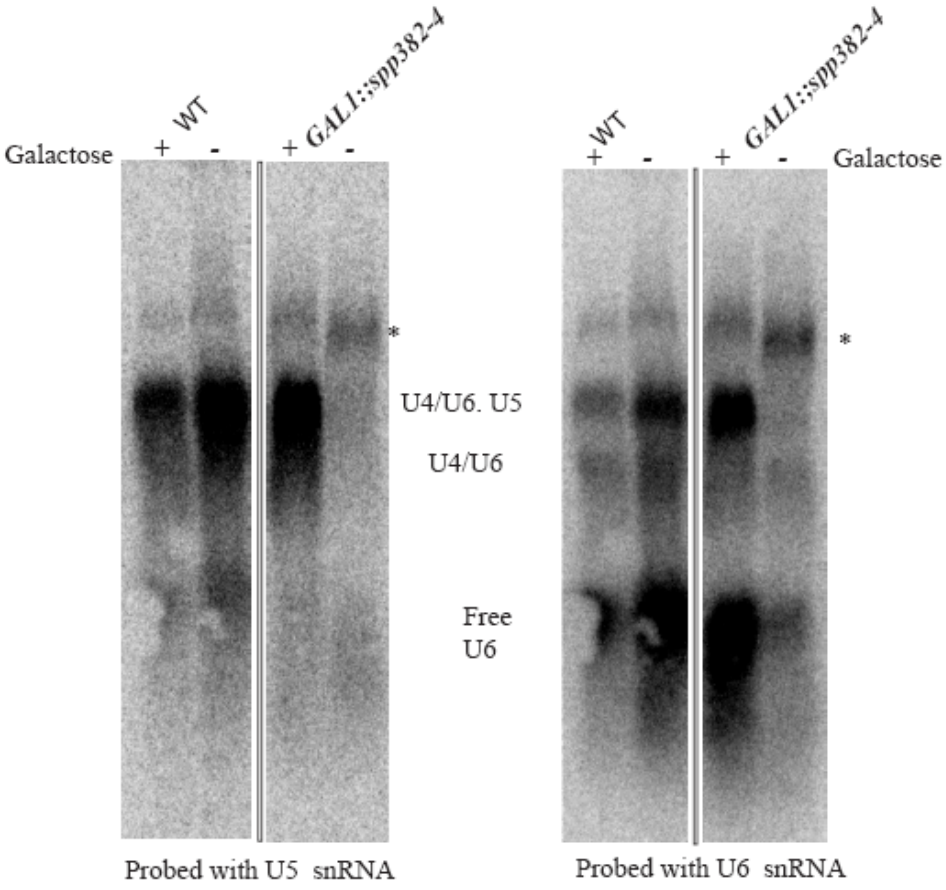

A
Total
TOP
SPP382
Grad.Fract. 1 3 5 7 9 11 13 15 17 19 21 23
U2>
U1>
U5L>
U5S>
U4>
U6>
U6
U4/U6
U5
U4/U6.U5
B
Total
TOP
GAL1::spp382-4, depleted
Grad.Fract. 1 3 5 7 9 11 13 15 17 19 21 23
U2>
U1>
U5L>
U5S>
U4>
U6>
U6
U4/U6
*
U4/U6.U5

I also monitored snRNP assembly by running native polyacrylamide gels with extracts that contain Spp382p or are depleted of the protein (see Material and Methods for details). After transfer, the blot was hybridized with radiolabeled snRNA-specific probes. Consistent with the glycerol gradient fractionation results, when Spp382p is depleted in vivo, tri-snRNP assembly is affected. With U5 snRNA specific probe, the Spp382p depleted lane shows little or no tri-snRNP, compared with the non depleted or wild type control (Fig 3.11, Panel A). When probed with either U5 or U6 snRNA specific probe, an aberrant complex (see * in the Figure 3.10, migrates more slowly compared to tri snRNP) is seen (Fig 3.10, Panel B). I also detected U2 in this complex when I probed the blot with a U2 specific probe (data not shown). This appears to be a stalled late spliceosome, with the U2.U5.U6 snRNAs. Taken together this indicates that depletion of Spp382p negatively affects tri-snRNP assembly or stability.

Spp382p binds spliceosomes including defective complexes that lack the 5' exon.

To investigate whether Spp382p associates with the splicing complex, I affinity purified a Tap-tagged derivative (Spp382-TAP) by IgG-agarose selection from in vitro assembled splicing complexes and assayed for co-recovery of the uniformly labeled substrate. As controls, extracts were also assayed from cells that express Prp19-TAP, a protein that stably binds the spliceosome right before

FIG 3.11. SnRNP profile of Spp382p depleted extracts. Splicing extracts were prepared from wild type cells or from *GAL1::spp382-4* strain grown on glucose (to effect metabolic depletion of Spp382p). The extracts were analyzed by native gel electrophoresis and northern blotting. The position of the tri-snRNP (U4/U6.U5), disnRNP(U4/U6) and free U6 are indicated. The * denotes a slowly migrating complex enriched after Spp382p depletion that might represent an aberrant spliceosome.


Galactose
+
-
WT
+
-
GAL1::spp382-4
*
U4/U6. U5
U4/U6
Free
U6
Probed with U5 snRNA
WT
+
-
GAL1::spp382-4
+
-
Galactose
*
Probed with U6 snRNA

or concurrently with U4 loss, and BBP-TAP, a protein that associates transiently with the spliceosome early in assembly.

The Prp19-TAP protein pulls down the lariat intermediate and excised intron and free 5' exon in similar relative amounts compared with the input sample (Fig. 3.12, panel A, lanes 13-18). A little unprocessed pre-mRNA is also recovered but no spliced mRNA is found associated with the Prp19-TAP, consistent with the very rapid dissociation of mRNA from the spliceosome. Only a minor amount of pre-mRNA, but no lariat intermediate or excised intron is recovered with a TAP tagged branchpoint binding protein (BBP). As expected with a protein that binds early and transiently to the splicing apparatus, the pre-mRNA signals decrease to background (WT no tag) with BBP-TAP, late in the splicing reaction (Fig. 3.12, panel A, lanes 7-12).

Both lariat intermediate and excised intron are recovered with Tap-tagged Spp382p (Fig. 3.12, panel A, lanes 1-6). When compared to the input RNA, the recovery of excised intron appears favored, especially late in the splicing reaction. Some unprocessed pre-mRNA but little or no spliced mRNA is found in the bound fraction. Unlike Prp19-TAP, little or no 5' exon signal is detected in the Spp382-TAP IgG pellet, even with a four-fold increase in exposure time (Fig. 3.12, panel A, compare lanes 4-6 and 28-30). The absence of the 5' exon is unexpected since this is not a natural form of the splicing complex.

To further investigate the binding of Spp382p to "5' exonless" complexes, I assayed a substrate truncated just upstream of the 3' splice site (Fig. 3. 12, panel B) for which the lariat intermediate and free exon accumulate as terminal

products (since no downstream exon exists). Nonspecific RNA binding to the resin is negligible (lanes 11-12) and, as before, lariat intermediate and 5' exon are recovered from the 30 minute splicing reaction with Prp19-TAP in amounts that approximate the 2.5:1 signal ratio expected based on nucleotide content (lanes 9, 10). With Spp382p, lariat intermediate is recovered as is some pre-mRNA, but, as before, little or no free 5' exon is found in the IgG pellet (lanes 1 and 2).

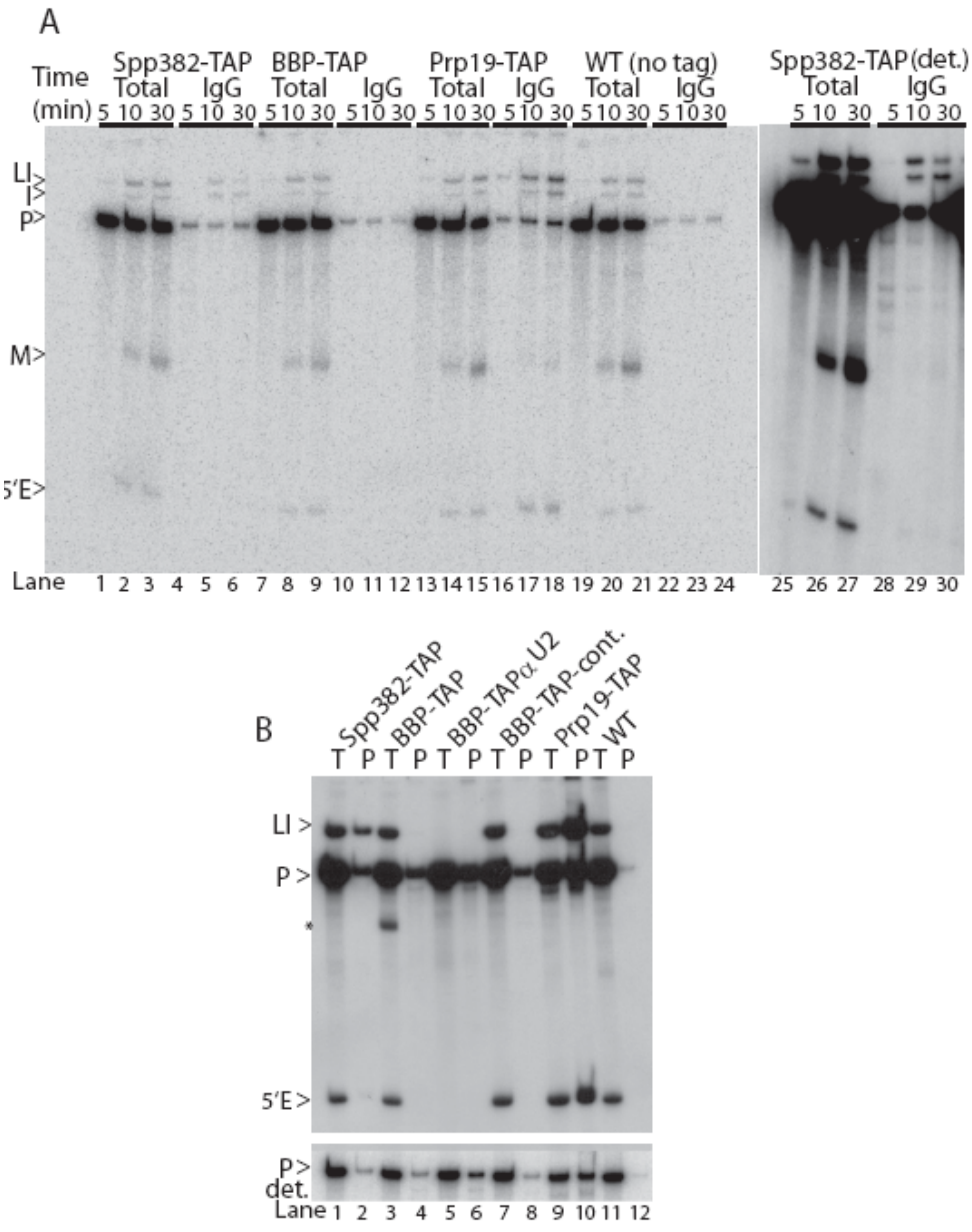
The trivial possibility that Spp382-TAP IgG selection causes release of the upstream exon appears unlikely as free (i.e., non-spliceosomal) 5' exon is resolved by glycerol gradient fractionation prior to selection (Figure 3.13 A). In addition, lariat intermediate molecules are preferentially recovered from lighter, possibly incomplete spliceosomal fractions (Fig 3.13, panels A and B). I note, however, that when complexes are first fractionated by glycerol gradient sedimentation more 5' exon is recovered with Spp382-TAP. The reason for this is unknown.

Less pre-mRNA is recovered with Spp382-TAP or with BBP-TAP than with Prp19-TAP (Fig. 3.12 B, compare lanes 2, 4, 10 with lane 12). Although the amount of pre-mRNA recovered with the TAP tagged proteins is low, it appears to be specific. For instance, the recovery is enhanced (and splicing blocked) in extracts where the BBP-TAP commitment complex is stabilized by oligonucleotide-directed RNase H digestion of U2 snRNA (compare lanes 4 and 6 in Fig 3.12 B, in the lower exposure detail). No change is observed if a control oligonucleotide is used (Fig 3.12 B lane 8). For Spp382-TAP, the pre-mRNA

recovered comes exclusively from the spliceosomal 40-50S fractions even though most of the pre-mRNA remains in the unassembled pool (Fig 3.13 panel A and B) again consistent with recovery from splicing complexes.

These results suggest that Spp382p may bind weakly or transiently to a subset of spliceosomal complexes, including complexes that have lost the 5' exon. To investigate the role of the upstream exon in sample recovery, I repeated the splicing reactions with *RPS17A* substrates where the 38 nucleotide 5' exon was lengthened (+13) or shortened by 16 or 25 nucleotides (Fig. 3.12 C). As measured by phosphorimaging, the lariat intermediate to fully excised intron (L/I) ratio, a measure of step two splicing inhibition, increases ~1.2-fold between the shortest and longest substrates (lanes 1–4). In contrast, this ratio more than doubles in the Spp382-TAP-recovered pool (lanes 5–8). This greater than proportionate increase reinforces the belief that a distinct subset of lariat intermediates, specifically the defective complexes, are being selected by Spp382-TAP. I note that based on the yield of RNA found in the IgG pellet, such complexes represent a very small percentage of lariat intermediate, less than 5% of the lariat intermediate present in the splicing reaction.

FIG 3.12. Spp382p binds defective spliceosomes. A. In vitro pre-mRNA splicing with ^{32}P-labeled *RPS17A* pre-mRNA processed for the indicated times under standard conditions (Pikielny, Rymond et al. 1986) and assayed by denaturing PAGE before (Total) or after IgG agarose selection (IgG). The detail (det.) to the right shows a 4-fold overexposure of the first 6 lanes to highlight the absence of upstream exon with IgG-agarose selected Spp382-TAP. The positions of the unprocessed pre-mRNA (P), lariat intermediate (LI), excised intron (I), spliced mRNA (M) and free upstream exon (5'E) are indicated.
B. In vitro splicing and RNA recovery with a 3' splice site truncated *RPS17A* mRNA blocked for the second step of splicing. Alternating lanes show the total processed RNA (T) and the IgG agarose pellets (P) after 30 minutes of splicing. The asterisk shows the position of linearized lariat intermediate sometimes observed under these conditions. The detail below (det.) shows a 4 fold underexposure of the corresponding pre-mRNA region to highlight the enhanced substrate recovery by IgG agarose after U2 snRNA degradation.
C. Extended (+13) or shortened (Δ16, Δ25) 5' exon *RPS17A* substrates were spliced for 30 min under standard conditions and then assayed for products before (lanes 1–4) or after (lanes 5–8) Spp382-TAP selection. The 5' exons (below the bar) were run on a second gel to resolve the smaller fragments.


A
Time
(min)
Spp382-TAP
Total
IgG
BBP-TAP
Total
IgG
Prp19-TAP
Total
IgG
WT (no tag)
Total
IgG
Spp382-TAP (det.)
Total
IgG
5 10 30
LI>
I>
P>
M>
5'E>
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
B
Spp382-TAP
BBP-TAP
BBP-TAPα U2
BBP-TAP-cont.
Prp19-TAP
WT
T P T P T P T P T P T P
LI >
P >
*
5'E >
P >
det.
Lane 1 2 3 4 5 6 7 8 9 10 11 12

C

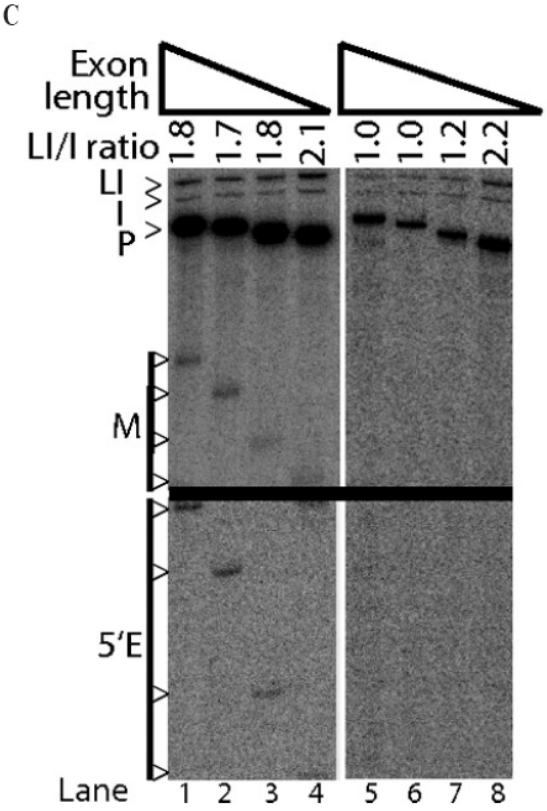
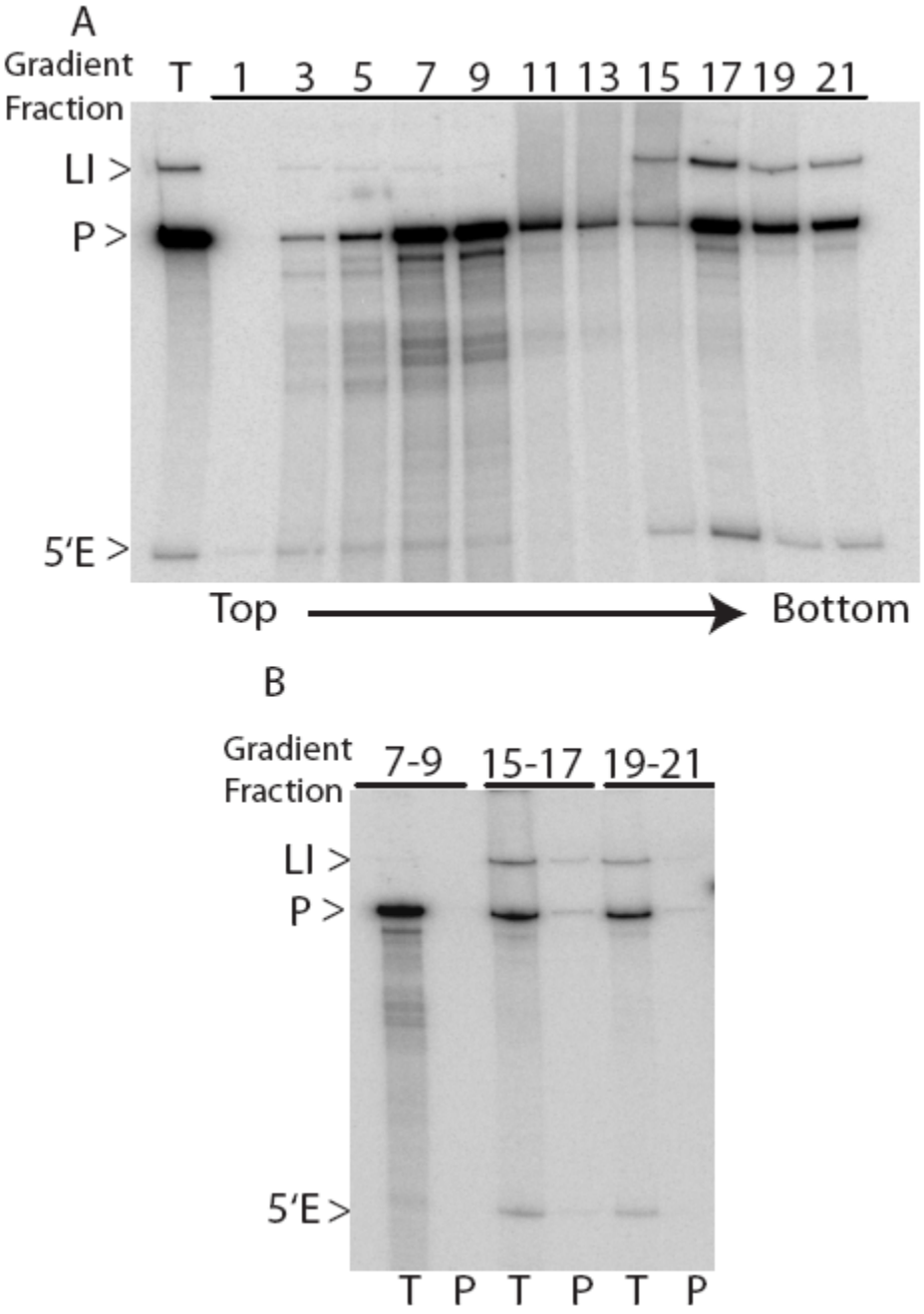

Exon
length
LI/I ratio
1.8
1.7
1.8
2.1
1.0
1.0
1.2
2.2
LI
I
P
M
5'E
Lane
1
2
3
4
5
6
7
8

FIG 3.13. Exon release occurs before Spp382-TAP selection in lighter spliceosomal complexes. After 30 min of splicing with the 3' splice site truncated *RPS17A* mRNA in the Spp382-TAP extract, complexes were resolved by velocity sedimentation in a 15-40% glycerol gradient. **A.** Alternate fractions were assayed on a denaturing polyacrylamide gel and the positions of the splicing precursor and intermediates are noted on the left.
B. The indicated gradient fractions were pooled, selected by IgG agarose, and resolved by denaturing PAGE as total, unselected RNA (T), or RNA enriched in the IgG agarose pellet (P).


A
Gradient Fraction
T 1 3 5 7 9 11 13 15 17 19 21
LI >
P >
5'E >
Top
Bottom
B
Gradient Fraction
7-9 15-17 19-21
LI >
P >
5'E >
T P T P T P

***Genetic interactions of Spp382p*.**

Chen et. al showed that Prp43p requires Spp382p for intron release from the spliceosome in vitro (Chan, Kao et al. 2003). This observation raises the interesting possibility that the suppression of *prp38-1* by mutants of *spp382* results from impaired spliceosome dissociation by Prp43p. If this is true, then the *prp38-1* stalled spliceosome must retain partial residual activity since suppression of a fully inactive complex would not be possible by this mechanism. Also one predicts that s*pp382*-based suppression would not be restricted to *prp38-1* but would extend to other defects in the spliceosome assembly pathway. Consistent with the first prediction, I find that overexpressing prp38-1p (the mutant protein) also suppresses the *prp38-1* strain similar to the previously characterized dosage suppressor, *SPP381* (Fig 3.14, 37 °C panel). This confirms that the prp38-1p spliceosomes have residual activity.

To test the second prediction, I scored additional conditional mutations within the components of the splicing enzyme for suppression. Like *prp38-1, prp38-2*, another mutant allele of *PRP38* (Xie, Beickman et al. 1998), is also suppressed by *spp382-4* (data not shown). I also tested *spp382* mutants' ability to suppress various other splicing mutants. I choose mutants which blocked the spliceosome at distinct steps in assembly, catalysis and disassembly. I find that the ts *prp8-1* mutant (blocked before activation) is suppressed by *spp382-4* (Fig 3.14) and also by the *spp382-1* and *spp382-6* alleles. *Spp382-6* also suppresses *prp19-1*, a single point mutant of Prp19p, which binds the spliceosome after or at the same time U4 snRNA leaves (Chan and Cheng 2005). No suppression is

observed with splicing mutants, *prp4-1* (spliceosomes in mutants blocked prior to catalysis)(Fig 3.15), *prp3-1* (mutants locked at pre-spliceosome stage), *prp6-1*(mutants blocked at pre-spliceosome stage), *rds3-1*(mutants blocked prior to U2 snRNP binding), (Wang and Rymond 2003) *prp39-1*(U1 snRNP binding fails in mutants), (Lockhart and Rymond 1994) when tested with either *spp382-4* and *spp382-6*. Likewise, no suppression was observed with mutant derivatives of two DExD/H-box proteins encoded by *prp16-1* (mutants blocked at step 1) and *prp22-1* (mutants fail to release message). Finally, in support of a functional association of Spp382p and Prp43p, I find that the *prp43R424A* mutation (Martin, Schneider et al. 2002) is synthetically lethal with the *spp382-4* and *spp382-6* (data not shown).

I also initiated an investigation into *spp382* mutants' ability to suppress weak substrate mutations. I transformed *spp382-1* and *spp382-6* with reporter plasmids (Rymond, Pikielny et al. 1990) which contain a *RPS17A* intron with either a wild type branchpoint (HZ18, UACUAAC), a strong branchpoint mutation (UACUAAC → UACUA**C**C, HZ10) or a weak branchpoint mutant (UACUAAC → U**C**CUAAC, HZ3). The transformants were patched on dropout (minimal) plates lacking uracil and the level of beta-galactosidase activity was checked by overlay assay (see Materials and Methods). As seen in Figure 3.15, both *spp382-1* and *spp382-6* suppress the weak branchpoint mutation as seen with increased beta galactosidase activity (compare color intensity in HZ3 panel). No suppression is seen with the stronger branchpoint mutation (HZ10) and neither do the mutants appear to alter the use of the wild type substrate (HZ18).

FIG 3.14. Genetic characterization of extragenic suppression. Yeast with ts *prp38-1*, *prp8-1* or *prp4-1* mutations were transformed with a plasmid to overexpress the mutant *prp38-1* allele (*GAL1*::*prp38-1*) or the previously characterized *SPP381* dosage suppressor (*GAL1*::*SPP381*). The *GAL1*::*spp382-4* suppressor was expressed in the *prp38-1*, *SPP382*::*Kan*^{R} background. The cultures were serially diluted and plated on yeast extract/peptone/galactose medium at the restrictive temperature for *prp8-1* (30°C) and *prp38-1* (37°C). To detect even minor changes in growth, a semi-permissive temperature of 30°C was used for *prp4-1*.

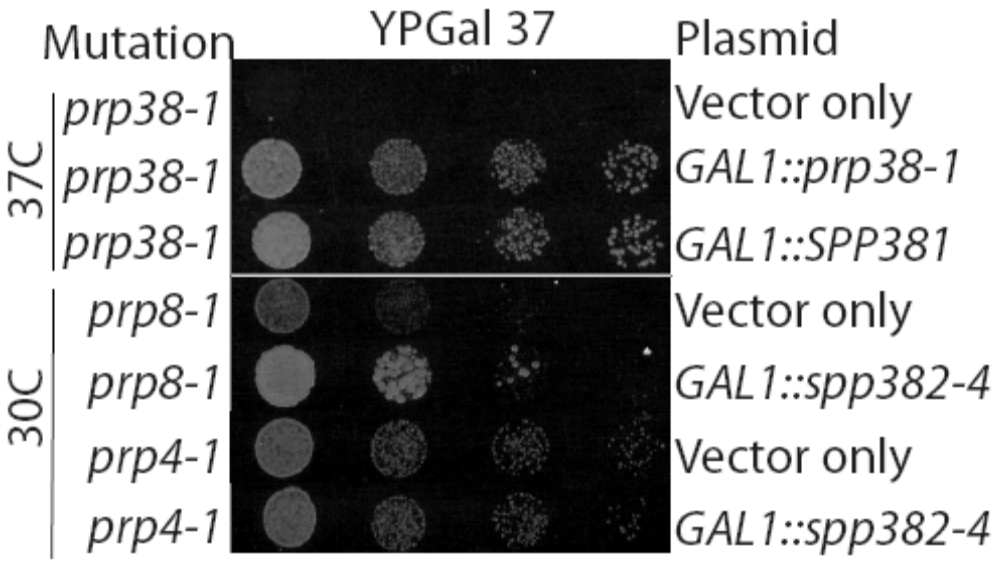
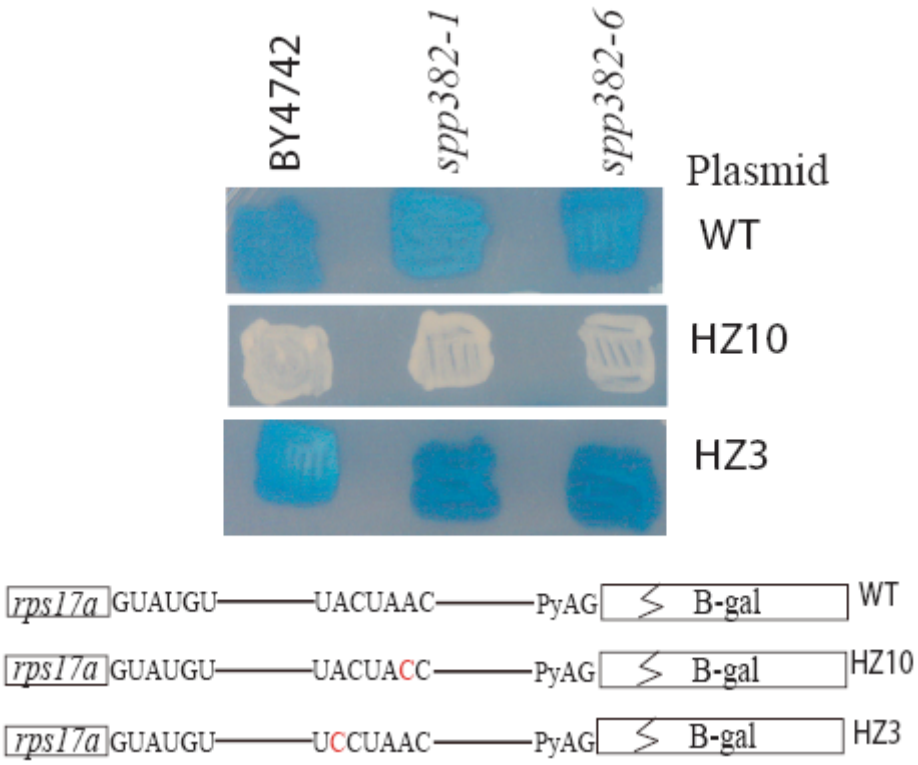

Mutation
YPGal 37
Plasmid
37C
prp38-1
Vector only
prp38-1
GAL1::prp38-1
prp38-1
GAL1::SPP381
30C
prp8-1
Vector only
prp8-1
GAL1::spp382-4
prp4-1
Vector only
prp4-1
GAL1::spp382-4

FIG.3.15. *spp382* alleles suppress weak substrate mutants. Wild type or *spp382* mutant strains were transformed with the indicated *RPS17a* reporter plasmid, grown on selective media for 3 days and then level of beta-galactosidase activity was checked by overlay assay.


BY4742
spp382-1
spp382-6
Plasmid
WT
HZ10
HZ3
rps17a GUAUGU——UACUAAC——PyAG B-gal WT
rps17a GUAUGU——UACUACC——PyAG B-gal HZ10
rps17a GUAUGU——UCCUAAC——PyAG B-gal HZ3

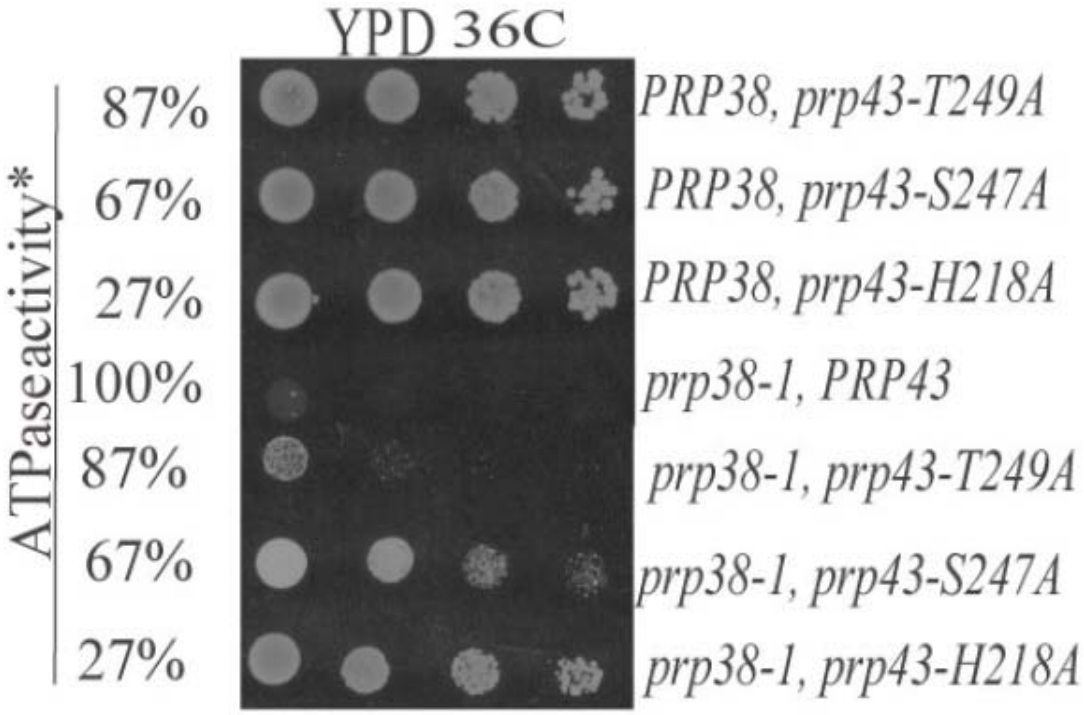
Lowered Prp43p ATPase activity suppresses* prp38-1 *defect.

To investigate whether *spp382* suppression is likely mediated via decreased Prp43p activity, I directly tested mutant *prp43* alleles for suppression of the *prp38-1* mutant. I obtained point mutants in the Prp43p DEAD/H box motif or in the SAT box motif from the Schwer lab (Martin, Schneider et al. 2002). These mutants differ in the level of residual ATPase activity. Among the three mutants tested, the DEAD/H box mutant (*prp43-H218A*) has the lowest ATPase activity (27%), while the SAT mutants (*prp43-S247A* and *prp43-T249A*) have 65% and 83% activity when compared to the wild type Prp43p activity (100%).

Each *prp43* mutant was tested for suppression of the *prp38-1* growth defect at 36C. The double mutants were serially diluted and plated on YPD plates at 36C. I found that each *prp43* mutant suppressed the *prp38-1* defect and the degree of suppression was inversely proportional to the reported ATPase activity (Fig 3.16). The mutant with the lowest ATPase activity (*prp43-H218A*) suppresses better than the prp43-*S247A* mutant which in turn is more effective than the weakest mutant, *prp43-T249A.*

FIG 3.16. *prp43* mutants also suppress *prp38-1* defect. Three mutant alleles of *PRP43* assayed for growth at 36°C in the *prp38-1* or wild-type (*PRP38*) background. The Prp43p ATPase activities are from Martin *et al* (2002).

It should be noted that the figure is a composite of multiple plate cultures incubated at the indicated temperatures for identical periods of time.



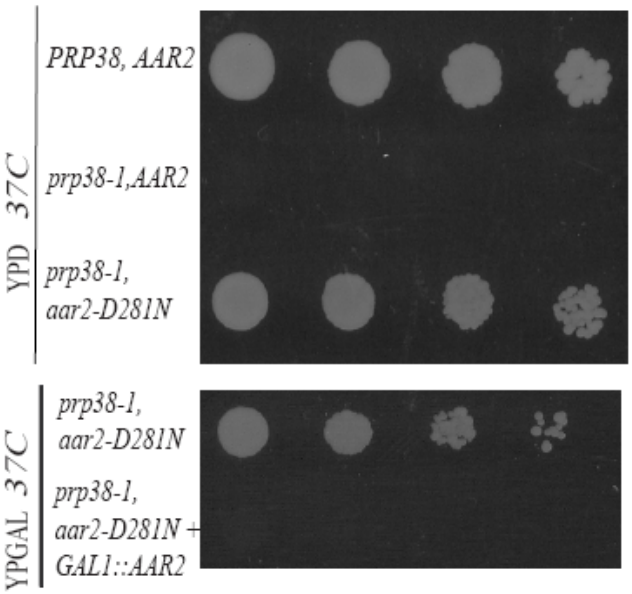
*Martin et. al (2002) J. Biol. Chem 277(20), 17743-50

A point mutant of Aar2p also suppresses the* prp38-1 *defect.

In order to identify the other strong recessive suppressor allele, I transformed the suppressor strain (E4) with 133 genes (Listed in Table 1 in Materials and Methods) that represent most of the known splicing factors as well as other genes involved in RNA processing or function. Transformants were selected by complementation of a plasmid-linked nutritional marker (*URA3*) at the permissive temperature (23 °C) and then screened by streaking representative colonies on nutrient rich media at 37 °C. One candidate gene (*AAR2*) was recovered that blocked E4 suppression (Figure 3.18, lower panel). To confirm that this was indeed a plasmid-linked event, the *GAL1::AAR2* plasmid was re-transformed into a naïve E4 suppressed strain. All the secondary transformants assayed showed the reacquisition of the temperature sensitive phenotype, demonstrating a plasmid-dependent event. Loss of plasmid by counter selection through growth in the presence of the anti-metabolite, 5-fluroorotic acid, resulted in colonies which were no longer ts (data not shown). Thus, Aar2p was most likely to be the suppressor allele. Sequencing of the mutant *aar2* locus revealed a single nucleotide change (G→A) that results in a single amino acid residue, D281N. Based on this, I conclude that Aar2p is the other suppressor allele.

FIG 3.17. *aar2-D281N* , a recessive point mutant, suppresses *prp38-1.*

Growth of wild-type yeast (*PRP38* and *AAR2*), the *prp38-1* mutant, and the suppressed *prp38-1*, *aar2-D281N* double mutant assayed after 2 days at 37°C in YPD. Overexpression of Aar2p inhibits growth of the double mutant (*prp38-1, aar2D281N*) as shown in the lower panel. Both strains were assayed at 37 C on YP-Gal after 2 days.


YPD 37C
PRP38, AAR2
prp38-1,AAR2
prp38-1,
aar2-D281N
YPGAL 37C
prp38-1,
aar2-D281N
prp38-1,
aar2-D281N +
GAL1::AAR2

Physical interactions of Spp382p with a DnaJ-motif protein, Cwc23p.

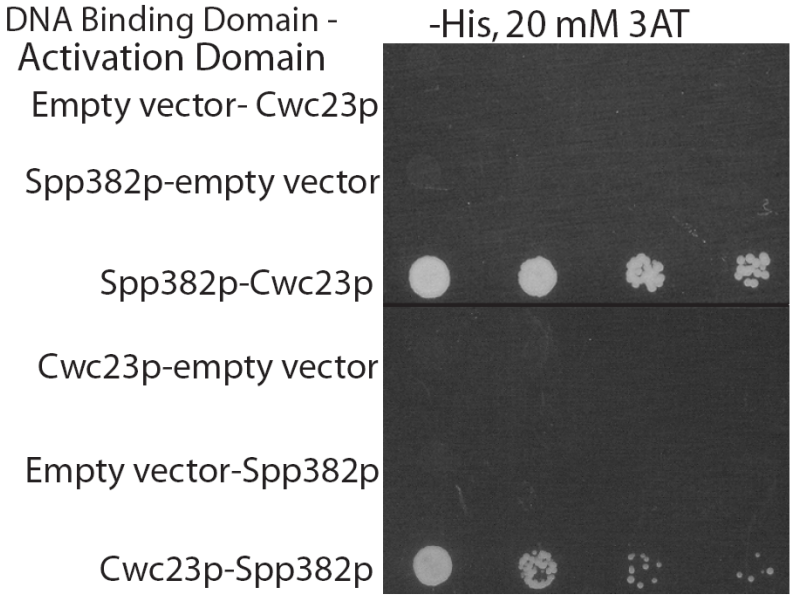
Spp382p has been reported in multiple complex particles recovered from yeast cells such as the penta-snRNP (Stevens, Ryan et al. 2002), the U4, U5, U6 snRNP spliceosomal complex (Wang, Hobbs et al. 2003) and other complexes generally including Prp43p (Gavin, Bosche et al. 2002; Tsai, Fu et al. 2005). However, since each of these purifications was done at low salt concentration, typically 100-150 mM NaCl, the complexity of the recovered multi-subunit particles prevents assessment of likely primary binding partners of Spp282p. To investigate high-affinity primary associations, I purified Spp382p-TAP at greater stringency (i.e. 450mM NaCl) and assayed for interacting proteins by mass spectroscopy. Cwc23p, a Dna J-like protein present in the spliceosome, is the only splicing related protein recovered with high confidence in this Spp382p-TAP complex (Table 3.2). The two ribosomal proteins and Rep2p (2 micron circle associated protein) were also identified and likely represent contaminants of this preparation.

The mass spectroscopy results suggest interaction between Cwc23p and Spp382p. To address this possibility by an independent methodology, I assayed full-length copies of Spp382p and Cwc23p for two-hybrid interactions. The yeast tester strain was grown for 3 days at 30 °C on plates lacking histidine and supplemented with 20 mM 3-aminotriazole to score for activation of the *GAL4*-regulated *HIS3* gene. As seen in Figure 3.18, Spp382p and Cwc23p show reciprocal positive interactions in this assay while neither binding partner auto-

Table 3.2. Spp382-Cwc23 interaction. List of proteins co-purified with Spp382-TAP identified by mass spectroscopy.

Spp382-TAP Proteins	MASCOT Mass Score	No. of Peptides identified
Cwc23p	114	3
Rpl14Ap	60	1
Rpl4Ap	27	1
Rep2p	23	1
Rrp5p	20	1

FIG. 3.18. Physical interaction of Spp382p with the spliceosomal DnaJ-like protein, Cwc23p. Serial dilutions of yeast stain PJ69-4a transformed with the indicated plasmids spotted on plates lacking histidine and supplemented with 3 aminotriazole and grown at 30 °C for 3 days.


DNA Binding Domain -
Activation Domain
-His, 20 mM 3AT
Empty vector- Cwc23p
Spp382p-empty vector
Spp382p-Cwc23p
Cwc23p-empty vector
Empty vector-Spp382p
Cwc23p-Spp382p

stimulates the yeast reporter gene when paired with an empty vector control. The two-hybrid results reinforce the interpretation of my proteomic data that the Spp38p splicing factor interacts with the uncharacterized Cwc23p spliceosome-associated protein.

Cwc23p was previously reported in splicing complexes purified with Cef1p (Ohi, Link et al. 2002) and co-purifies with the Prp43p-DExD/H-box ATPase (Gavin, Bosche et al. 2002) that acts with Spp382p to stimulate intron release from the spliceosome in vitro. Chen and co-workers did not detect Cwc23p associated with the Spp382p-Prp43p complex when assayed in vitro by western blotting (Tsai, Fu et al. 2005).

At the time of my observation, no direct evidence existed to show that Cwc23p actually plays an active role in the pre-mRNA splicing. In order to investigate the cellular function of Cwc23p, I mutated conserved residues within Cwc23p based on sequence alignment of the human, zebra fish, mouse, and *E.coli* homologs (Figure 3.19) In addition to these selected sites, I also randomly mutated the *CWC23* open reading frame using the Genemorph Kit from Stratagene. The plasmid-borne *CWC23* gene was mutated and then transformed into a yeast strain where the essential chromosomal copy of *CWC23* was replaced with the KAN^R gene (Winzeler, Shoemaker et al. 1999) and a *URA3*-linked wild type *CWC23* allele was present on a second plasmid. The activity of the mutant allele was determined after removal of the *URA3*-linked wild type copy by FOA selection. Yeast that failed to grow on FOA medium were considered to have an inactivating mutation in the targeted *CWC23* allele. Yeast

viable after FOA selection were considered to possess only the putative mutant copy of *cwc23* and were scored for growth defects at 19 °C, 25 °C, 30 °C, and 37°C. All mutant alleles were sequenced to confirm the existence of DNA changes.

The DNA J domain is an 80 amino acid motif (residues 15-95 in Cwc23p) (Sahi and Craig 2007). Several of the mutants presented here reside within this sequence (*cwc23- 1, -2* and *-4*) or in the case with the more complex mutants, at least one of the altered nucleotide lies within this domain (*cwc23-3,5,6,7*and *8*). Mutations within the highly conserved HPD sub region of the Cwc23p J-domain motif were found to impair growth (alleles *cwc23-1* and *cwc23-2*) (Figure 3.19, residues 50-52 marked in yellow and Figure 3.20). Mutant *cwc23-3* showed wild type growth at 37 °C but was cold sensitive and grew much less well at 30 °C or below (See Figure 3.20 and Table 3.2). A mutant in which amino acids 80-84 are deleted (*cwc23-4*) and *cwc23-6* (H65L, L136P, K147P) were somewhat less severe but likewise showed growth defects at both the low and high temperature. The *cwc23-5* mutant with 3 amino acid substitutions (L22F, L136P, K147R) grows slowly at all temperatures. Mutants *cwc23-7* and *cwc23-8* were lethal. For all the complex changes (*cwc23-3, 5, 6, 7, 8*), it is unclear whether it is changes in the J-domain or the mutations outside the J-domain or a combination of mutations in both result in the growth impairment or lethality.

FIG 3.19. Alignment of the Cwc23p homologs across species. The abbreviations used are, E.c – *Escherichia coli* (UniProt accession number P08622), D.r – *Danio rerio* (UniProt accession number Q7ZUW0), H.s – *Homo sapiens* (UniProt accession number Q96EY1), M.m- *Mus musculis* (UniProt accession number Q99M87), S.c - *Sacchromyces cereviciae*(UniProt accession number P52868).

```
M.m.   1 ---MAAWCSPRWLRVAVGTPRLPAAAGRGVQQPQGGVVATSLCRKLCVSAFGLSMGAHGP
57
H.s.   1 ---MAARCSTRWLLVVVGTPRLPAISGRGARPPREGVVGAWLSRKLSVPAFASSLTSCGP
57
D.r.   1 MACSAARSSARWISAAVSSSHSRACSPFIPRADGAYRGFKSLSGRQMWTTRGGLSGGAGA
60
E.c.     ------------------------------------------------------------
S.c.     ------------------------------------------------------------


M.m.   58 RALLTLRPGVRLTGTKSFPFVCTTSFHTSASLAKDDYYQILGVPRNASQKDIKKAYYQLA
117
H.s.   58 RALLTLRPGVSLTGTKHYPFICTASFHTSAPLAKEDYYQILGVPRNASQKEIKKAYYQLA
117
D.r.   61 AVTLSG-----MTAIGSSHVVCKMSFHTSAPSRKQDFYQILGVPRSATQKEIKKAYYQMA
115
E.c.    1 ------------------------------MAKQDYYEILGVSKTAEEREIRKAYKRLA
29
S.c.    1 --------------MPGHELEDVINQRLNLYDVLELPTPLDVHTIYDDLPQIKRKYRTLA
46
                                                :   .      :*:: *  :*

M.m.  118 KKYHPDTNKDDPKAKEKFSQLAEAYEVLSDEVKRKQYDAYGSAGFDPGTSSS-GQGYWRG
176
H.s.  118 KKYHPDTNKDDPKAKEKFSQLAEAYEVLSDEVKRKQYDAYGSAGFDPGASGS-QHSYWKG
176
D.r.  116 KKYHPDTNKEDPQAKEKFAQLAEAYEVLSDEVKRKQYDTYGSAGFDAGRAGAGHQQYWGG
175
E.c.   30 MKYHPDRNQGDKEAEAKFKEIKEAYEVLTDSQKRAAYDQYGHAAFEQGGMGG-----GGF
84
S.c.   47 LKYHPDKHPDNPSIIHKFHLLSTATNILTNADVRPHYDRWLIEFLRKTNDIE--------
98
           : *** :  : .   **  :  * : *::   *  ** :

M.m.  177 GPSVDPEELFRKIFGEFSSSP-FGDFQNVFDQPQEYIMELTFNQAAKGVNKEFTVNIMDT
235
H.s.  177 GPTVDPEELFRKIFGEFSSSS-FGDFQTVFDQPQEYFMELTFNQAAKGVNKEFTVNIMDT
235
D.r.  176 GTSIDPEELFRKIFGEFSGAQGFGDFNAIFNQPQEYVMELTFAQAAKGVNKEITVNIEGT
235
E.c.   85 GGGADFSDIFGDVFGDIFGGG-RGRQRAARGADLRYNMELTLEEAVRGVTKEIRIPTLEE
143
S.c.   99 ----RNKLIQKLEESESSTIPTTTPHPDLLQIQRHGELLRKLKHFNLPYGDWKHLNTQD-
153
                       ..                            :

M.m.  236 CERCDGKGNEPGTKVQHCHYCGGSGMETINTGPFVMRSTCRRCGGRGSIITNPCVVCRGA
295
H.s.  236 CERCNGKGNEPGTKVQHCHYCGGSGMETINTGPFVMRSTCRRCGGRGSIIISPCVVCRGA
295
D.r.  236 CQRCDGRGHEPGSKVQHCGNCNGTGMETVNTGPFVMRSTCRRCGGRGSVITSPCIACRGT
295
E.c.  144 CDVCHGSGAKPGTQPQTCPTCHGSGQVQMRQGFFAVQQTCPHCQGRGTLIKDPCNKCHGH
203
S.c.  154 ---------QENASQHPYYDCSTLRIVLDNFLQSNNKSNCLSHLR--------------
189
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```
M.m.  296 GQAKQKKRVTIPVPAGVEDGQTVRMP---------VGKREIFVTFRVQKSPVFRRDGADI
346
H.s.  296 GQAKQKKRVMIPVPAGVEDGQTVRMP---------VGKREIFITFRVQKSPVFRRDGADI
346
D.r.  296 GQTKQRKTVTVPVPAGIEDGQTVRMP---------VGKKEIFITFKVQKSPIFRRDGADI
346
E.c.  204 GRVERSKTLSVKIPAGVDTGDRIRLAGEGEAGEHGAPAGDLYVQVQVKQHPIFEREGNNL
263
S.c.  190 -----------------------------------NQVFITLSANEIYDIYFSERNN
211
                                             .: . .       :     :

M.m.  347 HSDLFISIAQAILGGTAKAQGLYETIN-VTIPAGIQTDQKIRLTGKGIPRINS-YGYGDH
404
H.s.  347 HSDLFISIAQALLGGTARAQGLYETIN-VTIPPGTQTDQKIRMGGKGIPRINS-YGYGDH
404
D.r.  347 HSDVMISVAQAILGGTIRAQGLYETIN-LSIPVGTQTDQRIRLSGKGIPRVSG-YGYGDH
404
E.c.  264 YCEVPINFAMAALGGEIEVPTLDGRVK-LKVPGETQTGKLFRMRGKGVKSVRG-GAQGDL
321
S.c.  212 YSKDDSIIIYTVFDTPITAQHVFRNWS---------------SGNLIPTVKDISPLIPL
255
            .    .  :  .       :                    *: :

M.m.  405 YIHIKIRVPKRLSSRQQNLILSYAEDETDVEGTVNGVTHTSTGGRTMDSS--AGSKDRRE
462
H.s.  405 YIHIKIRVPKRLTSRQQSLILSYAEDETDVEGTVNGVTLTSSGGSTMDSS--AGSKARRE
462
D.r.  405 YVHIKIKIPKMLTDRQRALMMSYAEDESDVEGTVNGVTSTTAGKRSAGN----------
453
E.c.  322 LCRVVVETPVGLNERQKQLLQELQESFGGPTGEHNSPRSKSFFDG--------------
366
S.c.  256 HYYSDFNLETELNDDIARLVSNEPILLD------------------------------
283
               .                 .
M.m.  463 AGEDNEGFLSKLKKIFTS 480
H.s.  463 AGEDEEGFLSKLKKMFTS 480
D.r.      ------------------
E.c.  367 ----VKKFFDDLTR---- 376
S.c.      ------------------
```

FIG.3.20. Growth characteristics of *cwc23* mutants. **A.** PCR-based mutagenesis of *CWC23*. The panel shows the amino acid changes and numbered coordinates on the 395 amino acid Cwc23 protein. **B.** Serial dilutions of *cwc23* mutants along with wild type yeast grown on YPD for 4 days at 25 °C and 2 days at 37 °C. It should be noted that the figure B is a composite of multiple plate cultures incubated at the indicated temperatures for identical periods of time.

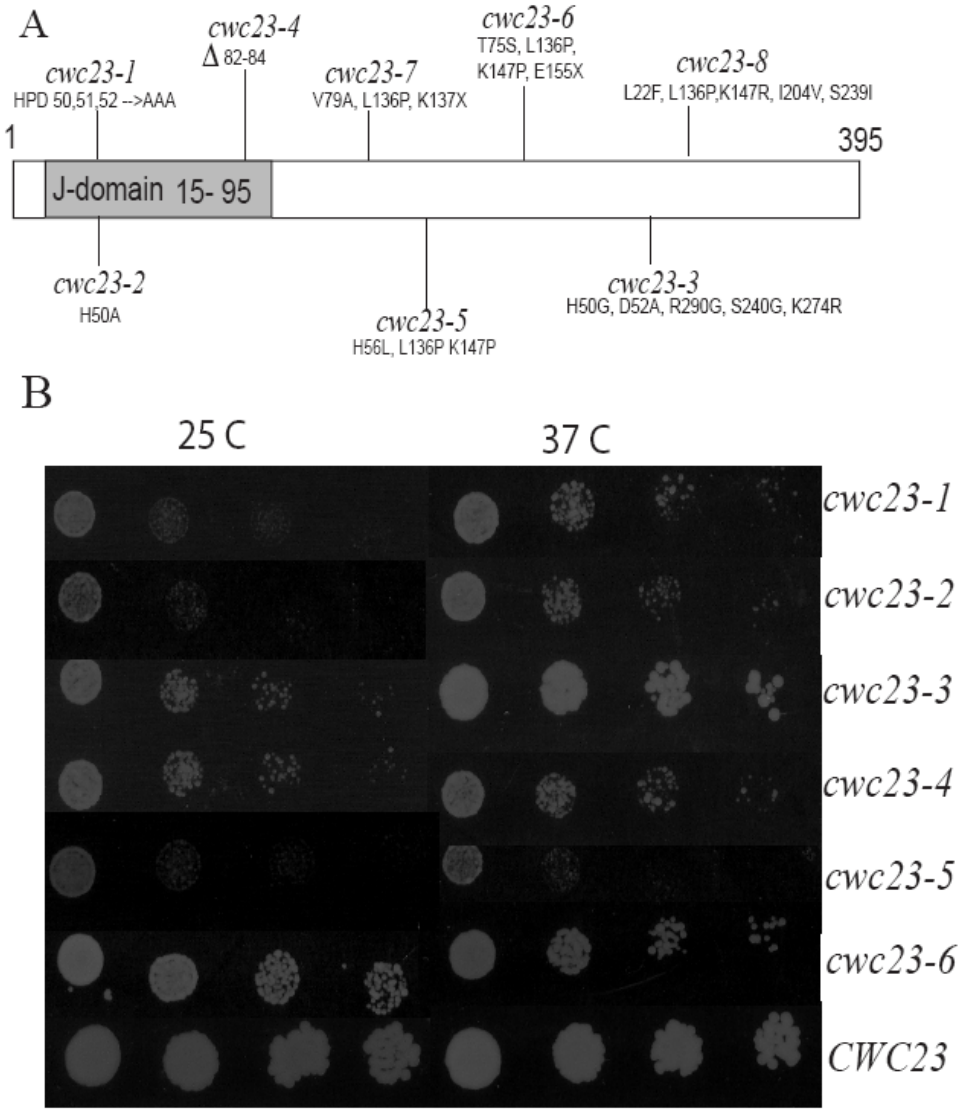

A
cwc23-1
HPD 50,51,52 -->AAA
cwc23-4
Δ 82-84
cwc23-7
V79A, L136P, K137X
cwc23-6
T75S, L136P,
K147P, E155X
cwc23-8
L22F, L136P,K147R, I204V, S239I
1
395
J-domain 15- 95
cwc23-2
H50A
cwc23-5
H56L, L136P K147P
cwc23-3
H50G, D52A, R290G, S240G, K274R
B
25 C
37 C
cwc23-1
cwc23-2
cwc23-3
cwc23-4
cwc23-5
cwc23-6
CWC23

Table 3.3. Growth phenotypes of *cwc23* mutants.

The yeast cells were grown for 7 days at 19 °C, 4 days at 25 °C, 3 days at 30 °C and 2 days at 37 °C.

Name	Change	Growth Phenotype @			
		19 °C	25 °C	30 °C	37 °C
Cwc23-1	HPD $_{50,51,52}$ →AAA	+/-	+/-	+	+
Cwc23-2	H50A	+/-	+/-	+	+
Cwc23-3	H50G, D52A, R209G, S240G,K247R	+/-	+	++	+++
Cwc23-4	H82, Y83, D84 deleted	+ +/-	+	+	+
Cwc23-5	L22F, L136P, K147R	+/-	+/-	+/-	+/-
Cwc23-6	H65L, L136P, K147P, E155X	++	++	++	++
Cwc23-7	V79A,L136P, K137X	Lethal			
Cwc23-8	L22F, L136P, K147R, I204V, S239I	Lethal			
CWC23	NA	+++			

Cwc23p is essential for normal cellular splicing.

Similar to what was shown above for *GAL1::SPP382*, incomplete metabolic depletion prevents the use of regulated *GAL1:: CWC23* to access the role of the protein in cellular splicing (i.e., this strain continues to grow on glucose-based medium; data not shown). Consequently, to investigate Cwc23p function in pre-mRNA splicing, I conducted northern analysis using RNA harvested from cells bearing the newly generated mutant alleles. To maximize the chances of seeing an effect, I used conditions designed to stress the cells at temperatures above or below the optimal growth temperature (of wild type yeast) at 30 °C. Specifically, I assayed yeast after temperature shift from 30 °C to 19 °C for 10 hours and after shift to 37 °C for 2 hours.

Splicing is impaired for both the J domain mutants (*cwc23-1* and *2*) and for *cwc23- 3* and *cwc23-4* as the *RPS17A* pre-mRNA/mRNA ratio increases relative to wild type yeast (Figure 3.21). I note that splicing is impaired for these mutants even at temperatures where growth inhibition is not clearly seen. The *cwc23-5* mutant was clearly splicing impaired at 36 while no splicing defects were obvious with *cwc23-6* mutant. Minor differences in the levels of excised actin intron were also observed although, in general, the elevated *ACT1* intron levels were more modest that what was observed with the *spp382* mutants.

Primer extension analysis was performed to investigate whether pre-mRNA or the similarly sized lariat intermediate accumulated after Cwc23p inactivation (Figure 3.22). Reverse transcription extension with an end-labeled *RPS17A* exon II oligonucleotide demonstrates primarily a step 1 block for

FIG. 3.21. **Cwc23p is essential for efficient cellular splicing.** Northern analysis of the non-lethal *cwc23* alleles along with the wild type yeast and the *prp38-1* mutant assayed at 19 C (-) or after 2 hours at 37 °C (+). The lower panel shows accumulation of the *ACT1* excised intron RNA.

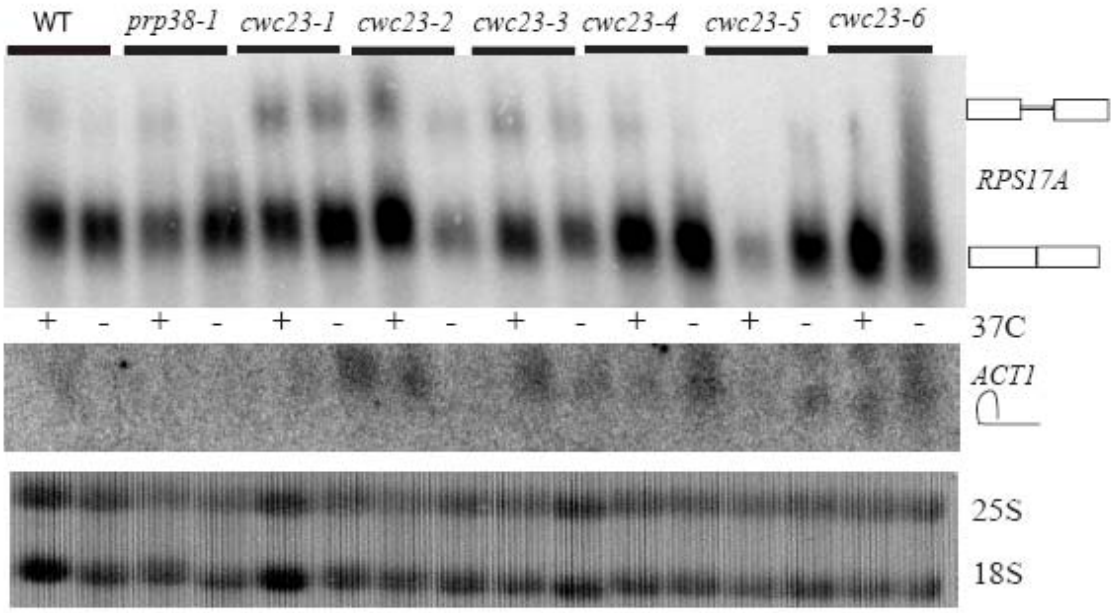
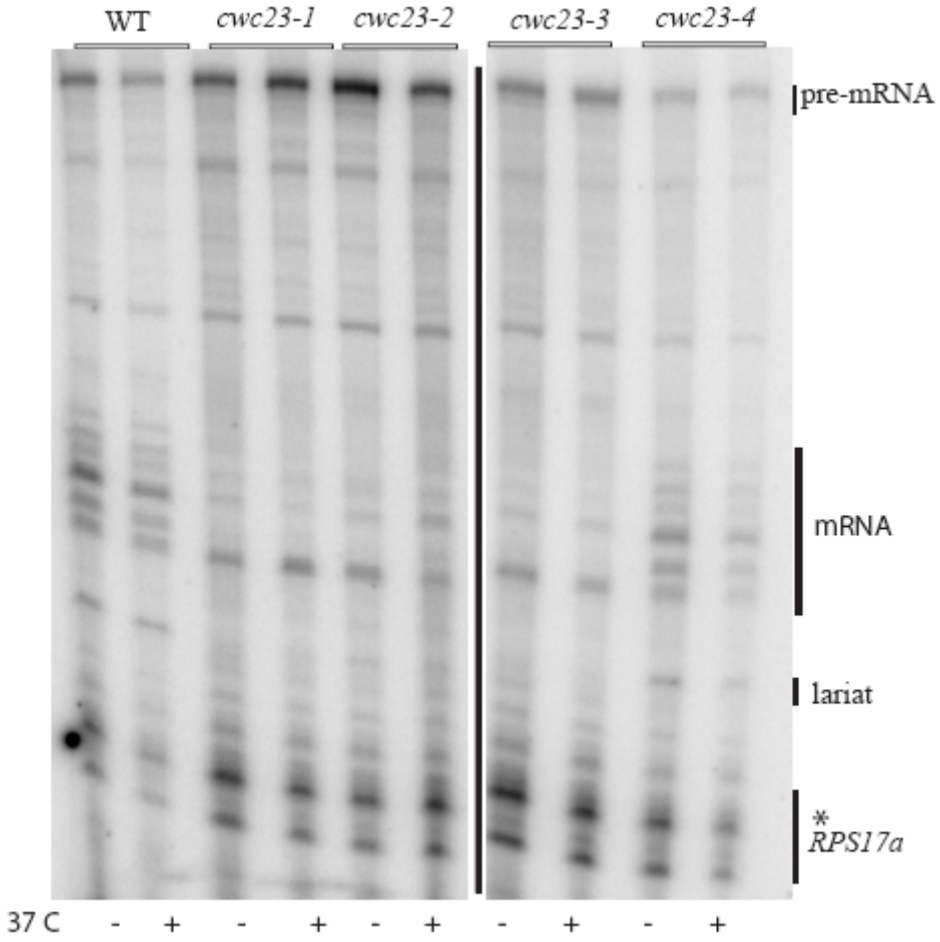

WT
prp38-1
cwc23-1
cwc23-2
cwc23-3
cwc23-4
cwc23-5
cwc23-6
RPS17A
+ - + - + - + - + - + - + - + -
37C
ACT1
25S
18S

FIG 3.22. *cwc23* mutants show inhibition of the first transesterification reaction in splicing. Primer extension analysis of RNA isolated from the indicated *cwc23* mutants and a wild type strain. Reverse transcription was conducted with oligonucleotide complimentary to exon 2 of the *RPS17a* reporter. The position of cDNA molecules corresponding to unspliced transcripts (pre-mRNA), spliced message (mRNA), lariat intermediate (lariat) and spliced endogenous *RPS17a* message (asterisk) is indicated to the right.


WT
cwc23-1
cwc23-2
cwc23-3
cwc23-4
pre-mRNA
mRNA
lariat
*
RPS17a
37 C
- + - + - + - + - +

alleles tested (i.e., *cwc23-1,-2, -3,* and *-4*) indicating that the protein plays a role in the first RNA cleavage ligation event in splicing. I cannot rule out a second role later in splicing for the Cwc23p, however, since a strong block at the first transesterification reaction would mask later defects in splicing.

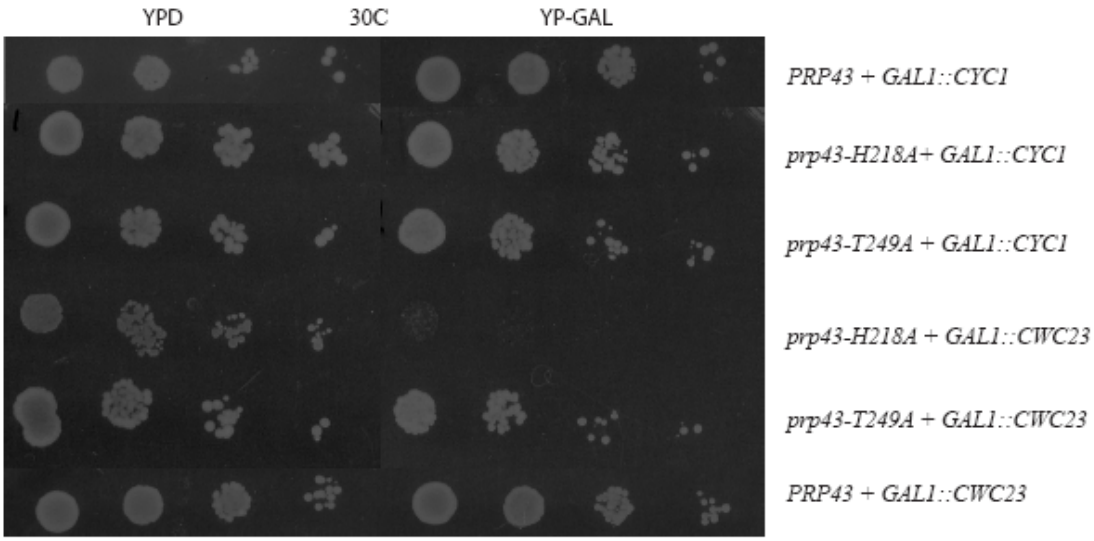
Genetic interactions of CWC23

I note that Cwc23p is the only J-domain protein reported to date in the spliceosome. The 80 amino acid J-domain motif was first defined in the *E. coli* DnaJ protein, a Hsp40-like protein in which the J domain plays a role in substrate presentation and or ATPase activation of an associated chaperone (e.g., Hsp70) (Qiu, Shao et al. 2006). DNA J motif proteins are involved in the assembly and disassembly events associated with a diverse set of multi-subunit complexes (Walsh, Bursac et al. 2004). In this light, the previously stated Spp382p- Cwc23p- Prp43p association is particularly intriguing as this defines possible associations with DExD/H-box recruitment factor (Spp382p), an enzyme (Prp43p) and possible regulator (Cwc23p). To explore this possibility, I overexpressed *CWC23* using the *GAL1* promoter in wild type yeast and in *prp43* mutant yeast with decreased Prp43p ATPase activity.

The *prp43* mutants transformed with a negative control plasmid (*GAL1::CYC1*) grow like the wild type strain on both glucose and galactose at 30 °C (figure 3.23). Overexpression of *CWC23* does not impair growth of a wild type strain either on glucose or galactose. While overexpression in the mutant, *prp43-H218A* (27 % ATPase activity; (Martin, Schneider et al. 2002)), impairs cell growth when grown on YP-galactose plates. This growth inhibition is reversed

FIG. 3.23. Overexpression of Cwc23p exacerbates the growth of *prp43* ATPase mutants. *Prp43* mutants were transformed with a plasmid to over express Cwc23p (*GAL1*::*CWC23*). The cultures were serially diluted and separately plated on either YP-Gal or YP-Dextrose medium at the permissive temperature for *prp43* mutants (30°C).

It should be noted that the figure is a composite of multiple plate cultures incubated at the indicated temperatures for identical periods of time.


YPD
30C
YP-GAL
PRP43 + GAL1::CYC1
prp43-H218A+ GAL1::CYC1
prp43-T249A + GAL1::CYC1
prp43-H218A + GAL1::CWC23
prp43-T249A + GAL1::CWC23
PRP43 + GAL1::CWC23

when the cells are grown on glucose. Overexpression of Cwc23p in the other *prp43* mutant, *prp43- T249A* (83 % ATPase activity; (Martin, Schneider et al. 2002), does not have any apparent effect on growth (Figure 3.23). This observation indicates that excess Cwc23p impairs cell growth when Prp43p ATPase activity is significantly reduced.

I showed above, within a certain range, reduced Spp382p activity or reduced Prp43p activity suppresses *prp38-1* and *prp8* splicing mutants (Figure 3.14 and 3.16). I also scored all the *cwc23* mutants for possible suppression of the *prp38-1* and *prp8-2* associated growth defects. (Figure 3.24 A and C). None of the *cwc23* mutants suppress the *prp38-1* growth inhibition at 36C or the *prp8-1* or the *prp8-2* temperature sensitivity at 30 °C. However, I do observe that *prp8-2* suppresses the growth defect of the J domain *cwc23* mutants at lower temperatures (*cwc23-1* and *2*). While both *cwc23-1* and *cwc23-2* mutants grow poorly at 19 °C, both strains grow better at this temperature when combined with *prp8-2*. This suppression can be reversed when a wild type *PRP8* is introduced into the strain (Fig 3.24 B) indicating that improved growth of the *cwc23* mutants is due to the *prp8* mutation. Thus, while providing genetic evidence for Cwc23p interaction with the conserved Prp8p protein that believed part of the spliceosome's active site (Grainger and Beggs 2005), this experiment did not reveal a suppressor phenotype for any of the *cwc23* alleles generated so far.

Simultaneous transformation of the Cwc23p and Spp382p as Gal4 fusion products lead to strong transactivation of the *HIS3* in the yeast two hybrid reporter strain, PJ694a. I tested whether the *cwc23* mutants showed impaired

FIG. 3.24. Genetic Interactions of Cwc23p. A. Serial dilutions of the double mutants along with the parents were spotted on yeast extract/peptone/dextrose medium and grown for 7 days in 19 °C or 3 days at 30 °C. **B**. Serial dilution of the double mutants along with or without the wild type *PRP8* grown on YPD at 19 °C for 10 days. **C.** Serial dilutions of the *cwc23, prp38-1*double mutants along with the parents spotted on YPD and grown for 3 days in 30 °C or for 2 days at 37 °C. It should be noted that the figures are a composite of multiple plate cultures incubated at the indicated temperatures for identical periods of time.

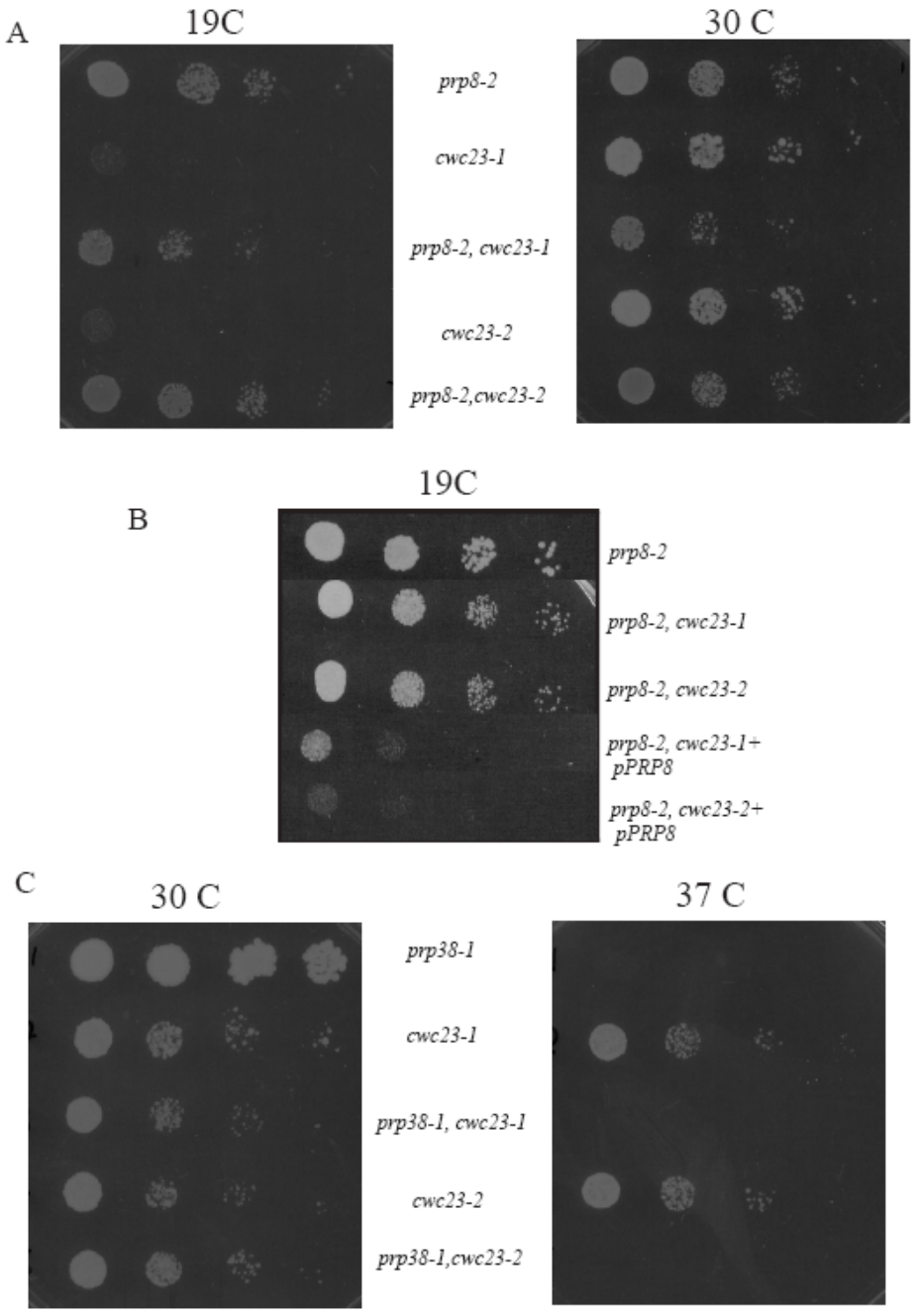

A
19C
30 C
prp8-2
cwc23-1
prp8-2, cwc23-1
cwc23-2
prp8-2,cwc23-2
B
19C
prp8-2
prp8-2, cwc23-1
prp8-2, cwc23-2
prp8-2, cwc23-1+ pPRP8
prp8-2, cwc23-2+ pPRP8
C
30 C
37 C
prp38-1
cwc23-1
prp38-1, cwc23-1
cwc23-2
prp38-1,cwc23-2

FIG. 3.25. Two hybrid interactions of *cwc23-1* and *cwc23-2* with Spp382p.

The yeast strain, PJ69-4a, bearing the indicated plasmids indicated below, was streaked on plates lacking histidine and supplemented with 3 aminotriazole and grown at 30 °C for 3 days.

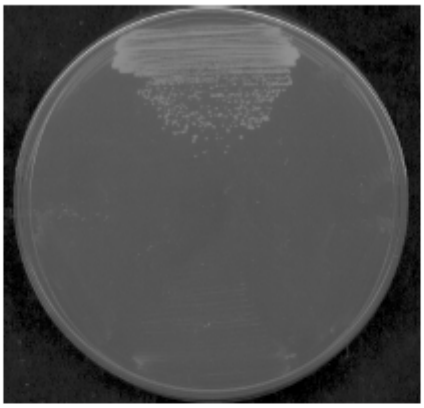
Panel A

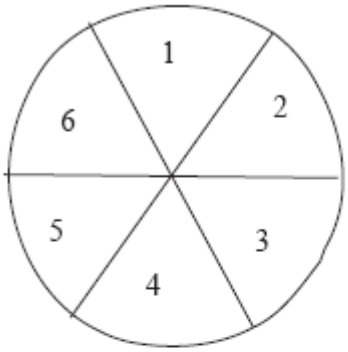
1. pACT::Cwc23p + pAS2 –Spp382p
2. pACT::Cwc23p
3. pAS2 –Spp382p
4. pACT-cwc23-1p + pAS2 –Spp382p
5. pACT::cwc23-1p
6. Empty

Panel B

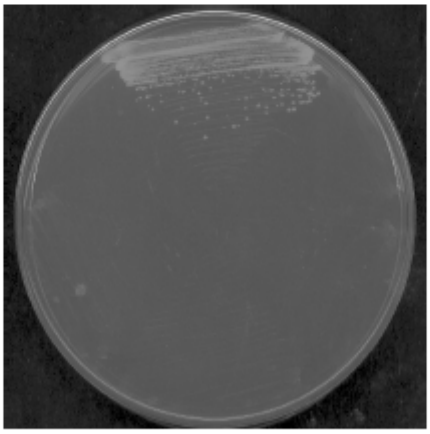
7. pACT::Cwc23p + pAS2 –Spp382p
8. pACT::Cwc23p
9. pAS2 –Spp382p
10. pACT-cwc23-2p + pAS2 –Spp382p
11. pACT::cwc23-2p
12. Empty

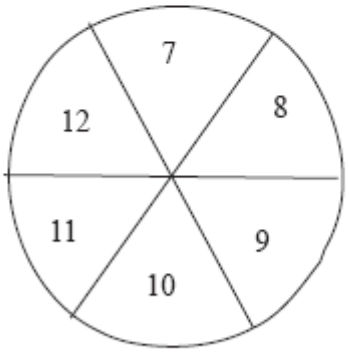
A





B





interaction with Spp382p. I tested Spp382p two hybrid interaction with cwc23-1p and cwc23-2p. For either mutant fused to the GAL4 activation domain (the pACT constructs, Figure 3.25 panel A -4 and B-10), I fail to see any colonies, indicating loss of interaction.

Overexpression of multiple different splicing factors inhibits growth of the spp382-1, prp38-1 double mutant suppressor strain.

I proposed that Spp382p mediates suppression due to impaired dissociation of the spliceosome via Prp43p. Based on this model, any change that enhances spliceosome turnover might be expected to counteract *spp382-1*-based suppression. To identify possible effectors of spliceosome turnover, the *GAL1* promoter was used to overexpress 133 yeast genes (See Materials and Methods), including most known splicing factors, in yeast in the original "suppressor" strain (that is, bearing both the *prp38-1* and *spp382-1* mutations) on rich medium containing galactose as sugar source, at 37 °C. While most genes did not influence growth, *PRP43*, *SPP382, CWC23*, and orf *YNL224c* (that I call *SQS1* for **sq**uelch of splicing **s**uppression) were among 14 genes found to impair growth relative to the empty vector control transformant (Figure 3.26 A). The remaining *GAL1* effectors encode a variety of splicing factors (Bud13p, Bud31p, Prp8p, Prp9p, Prp11p, Prp39p, Yhc1p), a protein involved in mRNA export and turnover, Dhh1p, and, surprisingly, mitochondrial ribosomal proteins Yml6p and

Mrp13p. The effect of overexpression on suppression was also checked at permissive conditions for *prp38-1* (i.e., 23 °C). Only *MRP13* and *SQS1* significantly impair growth of the suppressor strain at 23 °C on YP-galactose (Figure 3.26 B).

The *GAL1* promoter is transcriptionally induced 100 to 1000 fold by galactose. To learn if the growth defects observed required such high levels of induction, the transformants were assayed for growth on rich glucose-based media at 23 C and 37 °C. None of the effector genes altered growth relative to the control strain on YPD at 23 °C (data not shown). *GAL1* repression on glucose causes the *GAL1::PRP9* and *GAL1::MRP13* transformants to reacquire the suppressed (i.e., better growth) phenotype (Fig. 3.26 A) at 37 C. This is not true for most transformants, however, indicating that even a modest increase in effector protein level reduces *spp382-1* efficacy as a suppressor. Unexpectedly, the *GAL1::PRP43* and *GAL1::BUD13* transformants appear more effective in this assay when yeast are cultured on glucose. That is, the strains grow less well on glucose-based medium than on the galactose-based medium.

To investigate whether the impaired growth observed with overexpression required the double mutant (i.e., *prp38-1, spp382-1*) background I checked the growth of the single mutant parental strains and in wild type yeast. Other than the exceptions noted below, none of these genes significantly alter growth of the *spp382-1* or *prp38-1* single mutants when assayed on rich medium (Figure 3.26B). Overexpression of Prp43p-associated protein Ntr2p (Tsai, Fu et al. 2005) relieves the modest ts defect of the *spp382-1* single mutant (Figure

3.27B). As expected, overexpression of *PRP38* and *SPP381* enhance growth of the *prp38-1* single mutant (Figure 3.27 B) with minor impact on the (*prp38-1, spp382-1*) suppressor strain (Figure 3.26 B). *SPP381* does not suppress the *prp38-1* defect when assayed on glucose medium, indicating that significantly increased protein levels are needed to suppress this mutant strain ((Blanton, Srinivasan et al. 1992; Xie, Beickman et al. 1998). None of these genes affect the growth of wild type strain when assayed on rich medium.

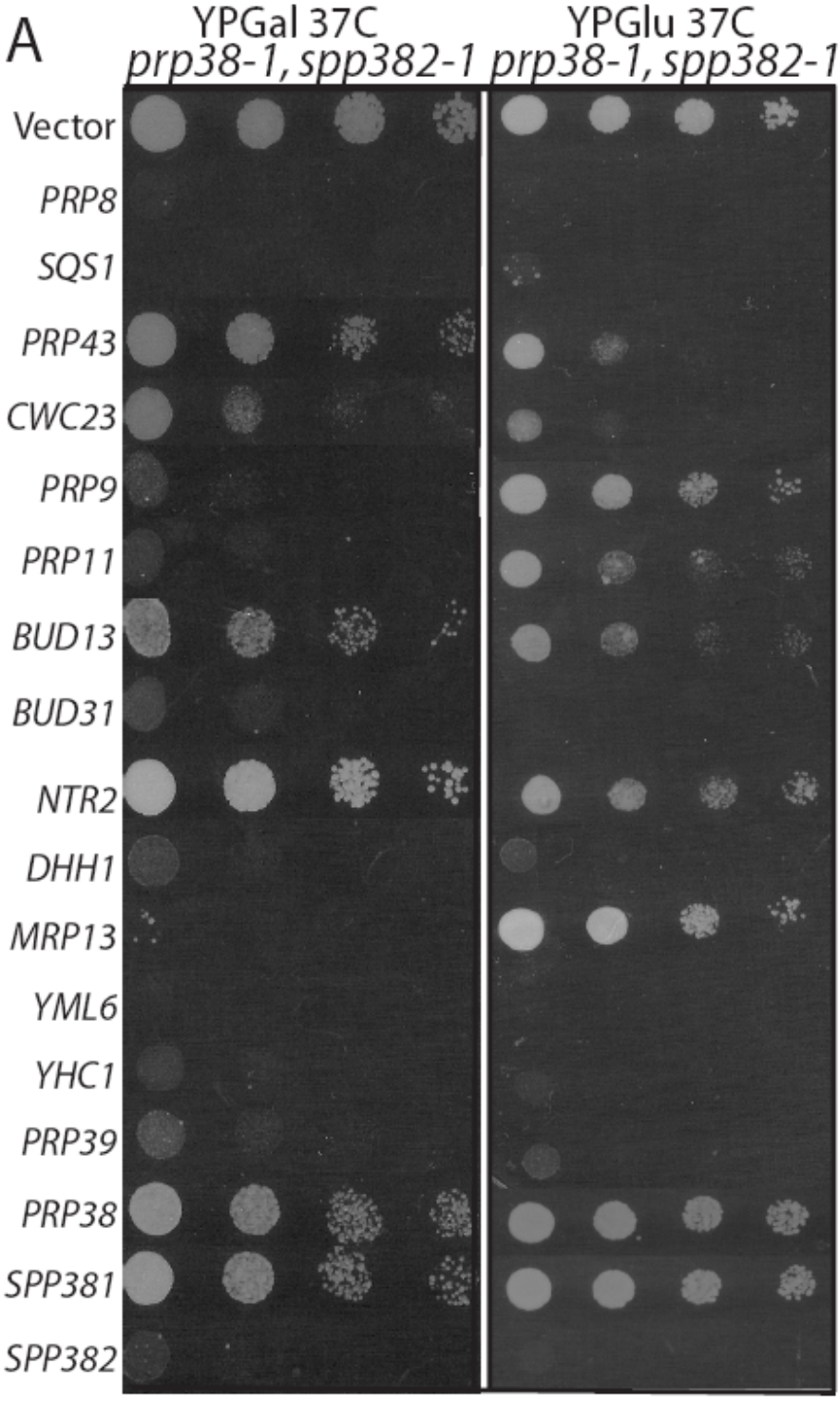
YNL224C/SQS1 overexpression was reported to impair cell cycle progression (Stevenson, Kennedy et al. 2001). Yet, we see no obvious growth defect when this gene is expressed in wild type yeast on YP-galactose medium. To investigate this obvious discrepancy, we reassayed growth of the transformants on selective minimal media (lacking uracil and with galactose or glucose as sugar source), the conditions used by the earlier group. Overexpression of all constructs except *SPP381* or *PRP38* impairs growth of the double mutant at 37. In addition, for *PRP8, BUD31, MRP13, YML6, YHC1, PRP39* and *SPP382,* the growth inhibition is reversed when grown on the drop-out glucose medium (Figure 3.26 C). Overexpression of *SQS1, DHH1, PRP8, PRP43, BUD13, BUD31, YML6* and *SPP382* also inhibit growth of the double mutant at 23 °C in galactose while none of the constructs show impaired grown on glucose at 23 °C.

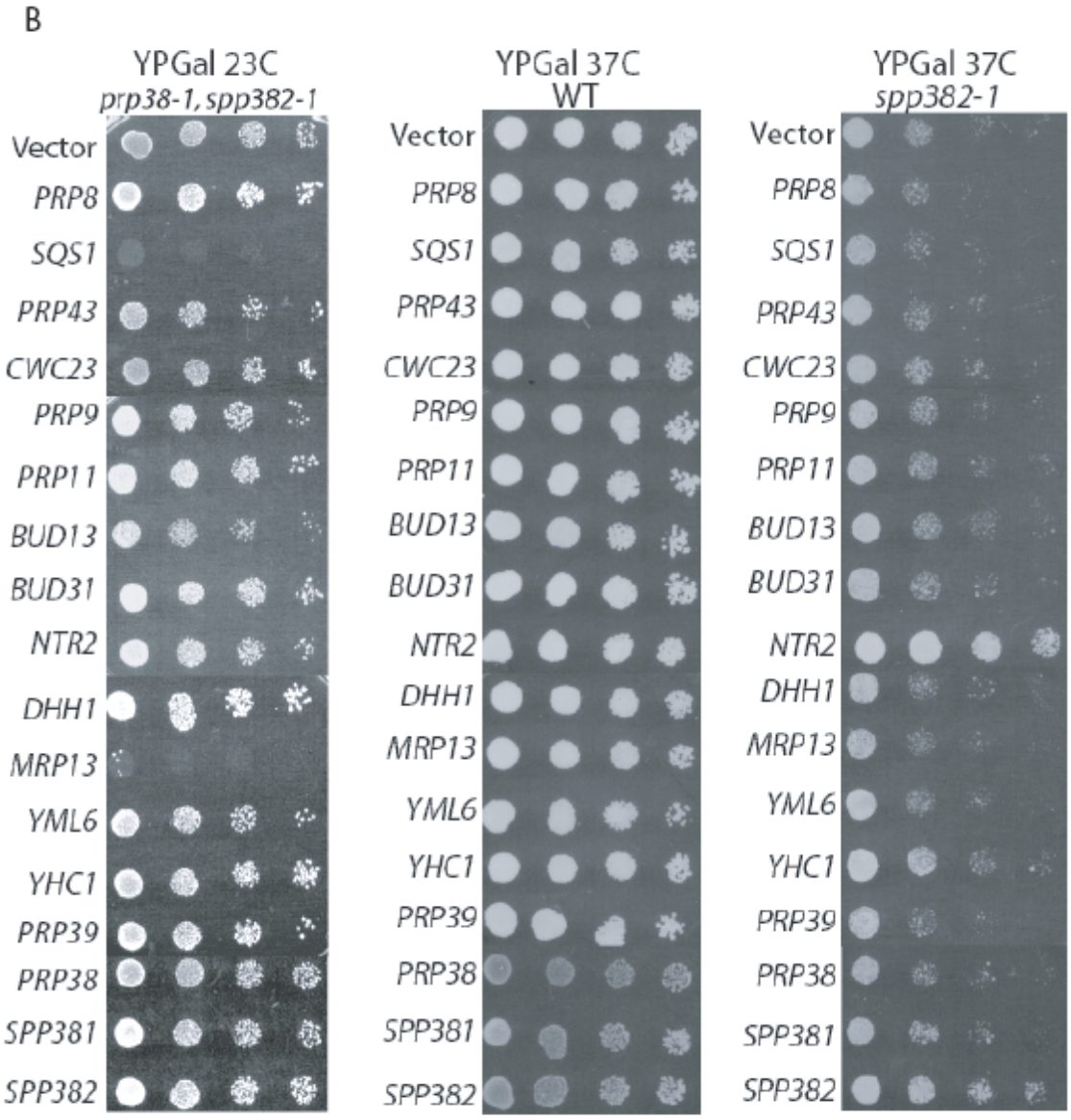
FIG 3.26. Impact of overexpression under different conditions. A. Double mutant (*prp38-1, spp382-1*) yeast cultures transformed with the indicated *GAL1* fusion genes were serially diluted and spotted at 37 °C on medium to induce (galactose) or repress (glucose) the *GAL1* promoter.

B. The *prp38-1, spp382-1* double mutant, wild type yeast (WT), the *spp382-1* single mutant, and the *prp38-1* single mutant strain transformed with either the control (*GAL1::CYC1*) or the indicated *GAL1* driven plasmids and assayed for growth at permissive temperature (23 C or 35C) or restrictive temperatures in YPGal or glucose based medium. The cultures were grown in parallel in the same experiment as Fig A. The far end of permissive temperature (35C) was selected for *prp38-1* to increase the chance of observing minor exacerbations of temperature sensitivity.

C. The *prp38-1, spp382-1* double mutant and wild type yeast (WT), transformed with either the *GAL1::CYC1*or the *GAL1* driven constructs and assayed for growth at permissive temperature (23 C) or restrictive temperatures in media lacking uracil, with either galactose or glucose as the sole source for sugar. The cultures were grown in parallel in the same experiment as Fig A.

It should be noted that the figures are a composite of multiple plate cultures incubated at the indicated temperatures for identical periods of time.


A
YPGal 37C
prp38-1, spp382-1
YPGlu 37C
prp38-1, spp382-1
Vector
PRP8
SQS1
PRP43
CWC23
PRP9
PRP11
BUD13
BUD31
NTR2
DHH1
MRP13
YML6
YHC1
PRP39
PRP38
SPP381
SPP382


B
YPGal 23C
prp38-1, spp382-1
YPGal 37C
WT
YPGal 37C
spp382-1
Vector
PRP8
SQS1
PRP43
CWC23
PRP9
PRP11
BUD13
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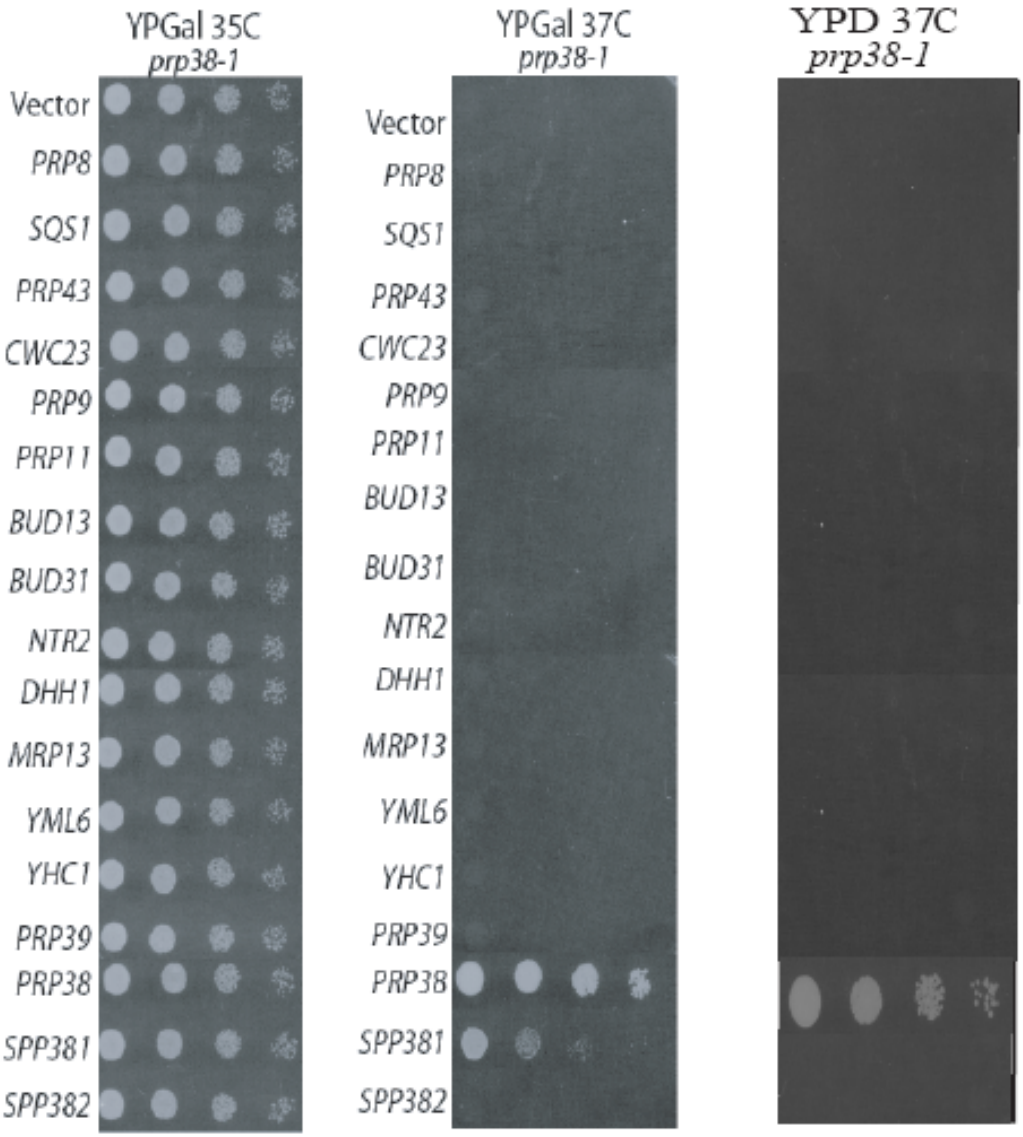
NTR2
DHH1
MRP13
YML6
YHC1
PRP39
PRP38
SPP381
SPP382

B


YPGal 35C
prp38-1
YPGal 37C
prp38-1
YPD 37C
prp38-1
Vector
PRP8
SQS1
PRP43
CWC23
PRP9
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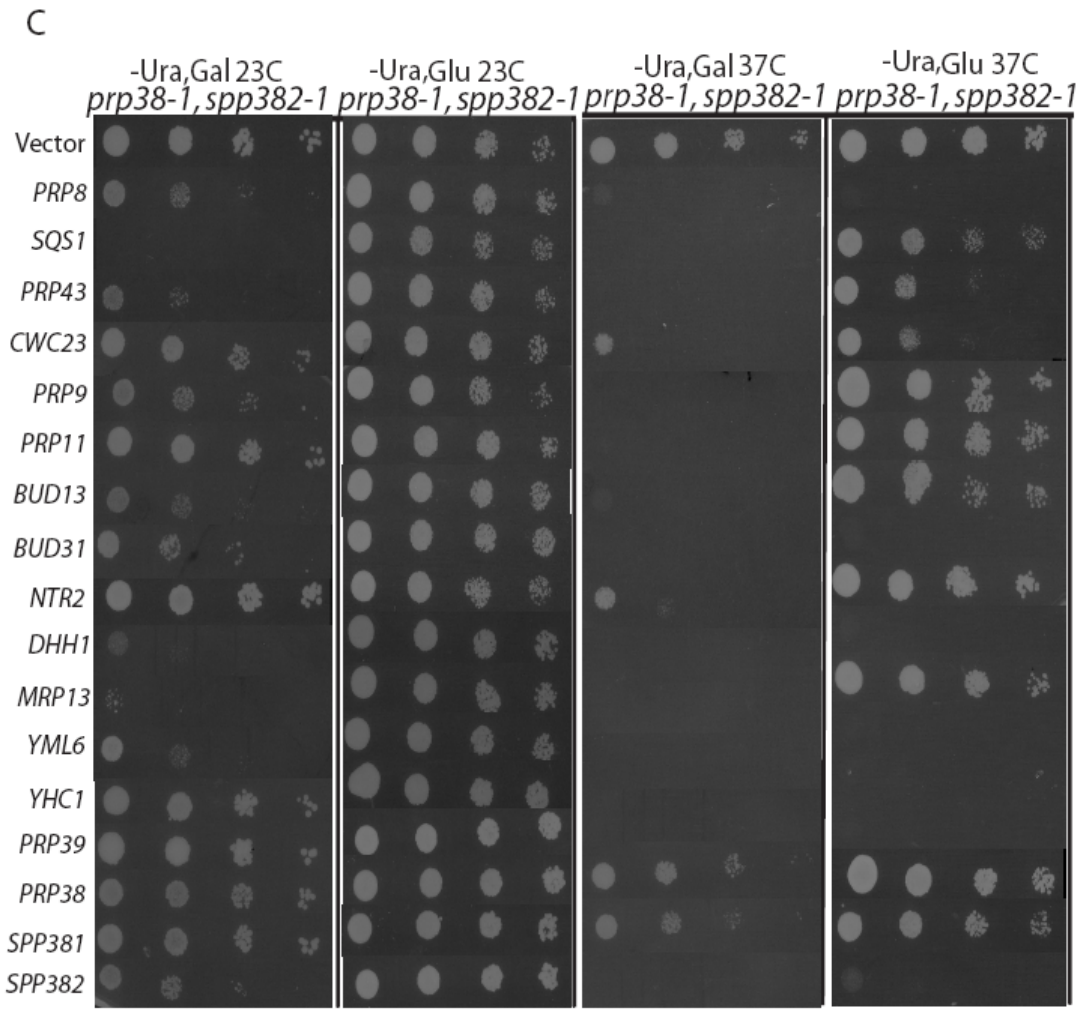
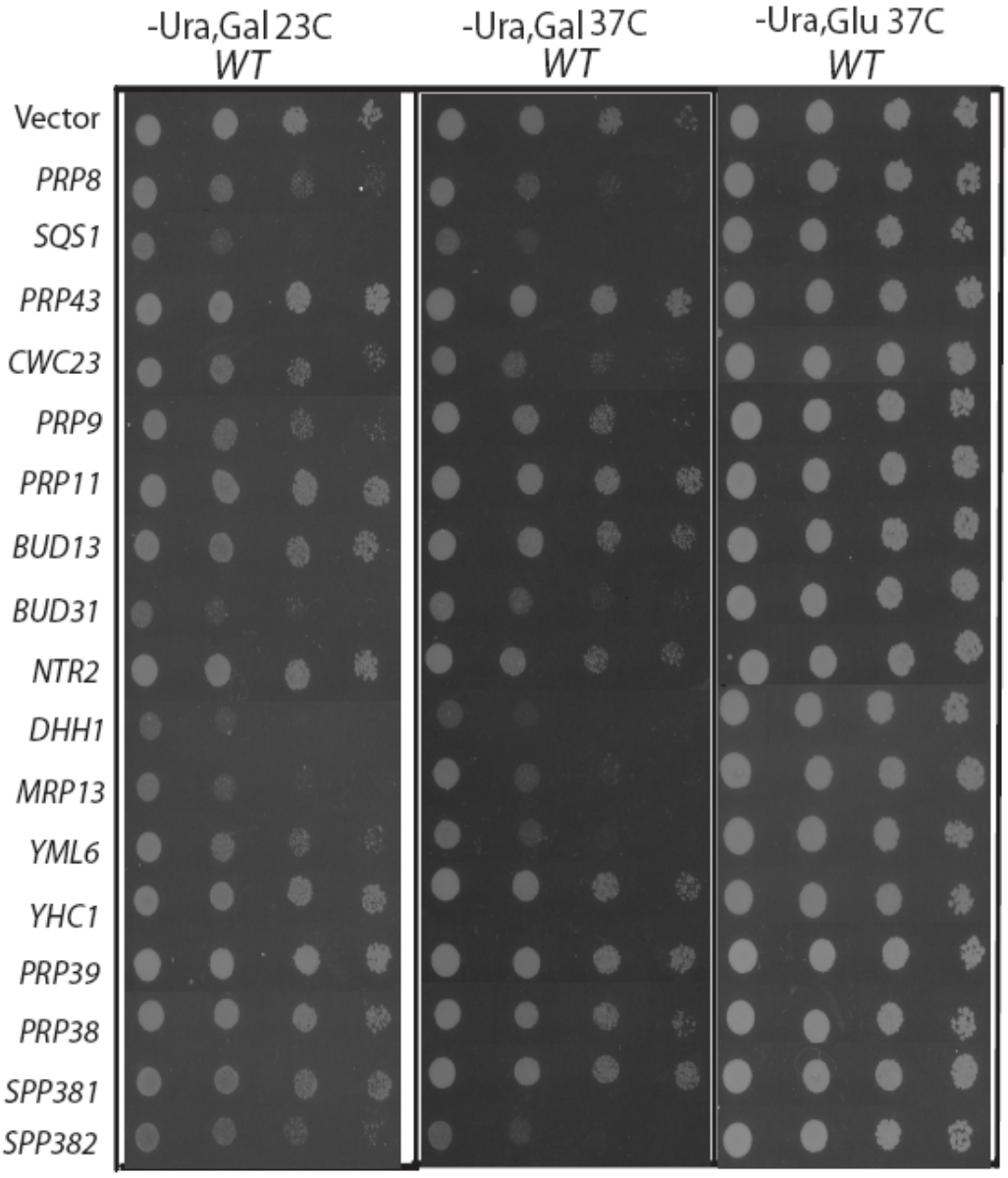
YML6
YHC1
PRP39
PRP38
SPP381
SPP382


C
-Ura,Gal 23C
prp38-1, spp382-1
-Ura,Glu 23C
prp38-1, spp382-1
-Ura,Gal 37C
prp38-1, spp382-1
-Ura,Glu 37C
prp38-1, spp382-1
Vector
PRP8
SQS1
PRP43
CWC23
PRP9
PRP11
BUD13
BUD31
NTR2
DHH1
MRP13
YML6
YHC1
PRP39
PRP38
SPP381
SPP382

C


-Ura,Gal 23C
WT
-Ura,Gal 37C
WT
-Ura,Glu 37C
WT
Vector
PRP8
SQS1
PRP43
CWC23
PRP9
PRP11
BUD13
BUD31
NTR2
DHH1
MRP13
YML6
YHC1
PRP39
PRP38
SPP381
SPP382

Unlike the case for rich medium, overexpression of *SQS1, BUD31, DHH1,* and *MRP13* also inhibits growth of the wild type yeast strain at 30°C and 37°C when cultured on the defined medium. Overexpression of *PRP8, CWC23, YML6* and *SPP382* impairs growth of the wild type at 37°C. The growth inhibition in these genetic backgrounds is reversed in all cases when the transformants are grown in selective glucose medium. Based on this, I conclude that overexpression of some splicing factors hypersensitizes the *spp382* mediated suppression and in some cases impairs growth of otherwise wild type yeast. Finally, for all the constructs, I confirmed that transformants revert to the original growth state after plasmid removal (data not shown).

Over expression of Sqs1p impairs cellular pre-mRNA splicing.

As noted above, overexpression of Sqs1p can impair growth of even the wild type yeast. In addition, I note that like Spp382p, Sqs1p is a G-patch protein (see figure 3.27) The increased sensitivity of the suppressed strain (*prp38-1, spp382-1*) to Sqs1s overexpression and the fact that Sqs1p can be affinity selected with complexes containing the Prp43p-DExD/H-box factor (Gavin, Bosche et al. 2002), suggest possible involvement of Sqs1p in splicing. To investigate this, I analyzed pre-mRNA splicing in wild type yeast grown in drop out glucose media (lacking uracil) and various times after shift to galactose (Figure 3.28 A) to induce *GAL1::SQS1*. While the pre-mRNA- message ratio (indicator of splicing efficiency) is similar to control cells when grown in glucose, the value increases as the time in galactose increases. Similar results were

obtained when Sqs1p overexpression was assayed in the *prp38-1* single mutant or the suppressed double mutant *prp38-1, spp382-1* (data not shown). The control which lacks *GAL1::SQS1* shows no inhibition of splicing when grown in galactose media. Under this condition no change is seen with the intronless *ADE3* gene. While splicing is inhibited, over expression of Sqs1p does not affect the stability of the splicing associated snRNAs. Northern analysis of the snRNAs shows that while splicing is impaired, the snRNAs are not degraded (Figure 3.28 C).

The *SQS1* gene is not essential for yeast viability (SGD database) and I observe no growth defect in a *sqs1::KAN* knockout strain when grown at RT or at 37 °C (Figure 3.30). While the overexpression results suggest splicing involvement, northern analysis of RNA isolated from a *sqs1::KAN* null mutant shows no obvious defect in splicing under normal (RT) or elevated temperature (37 °C) (Figure 3.28 B). Thus, while overexpression of Sqs1p is cytotoxic and inhibits splicing, the absence of this protein does not significantly impair pre-mRNA splicing.

FIG. 3.28. G-patch alignment of Spp382p and Sqs1p. Alignment of G-patch motif with the consensus below. The sequences are named using the notation Protein name _ Species abbreviation_Gene Bank GI (Gene identification number). Abbreviations used Sc - *Saccharomyces cerevisiae*, Hs - *Homo sapiens*, Sp -*Schizosacchromyces pombe*, Ca -*Candida albicans*, At - *Arabidopsis thaliana*.

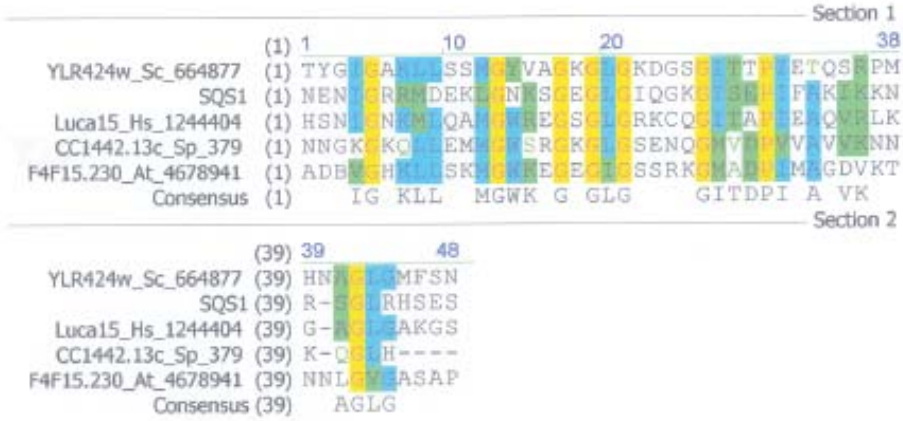
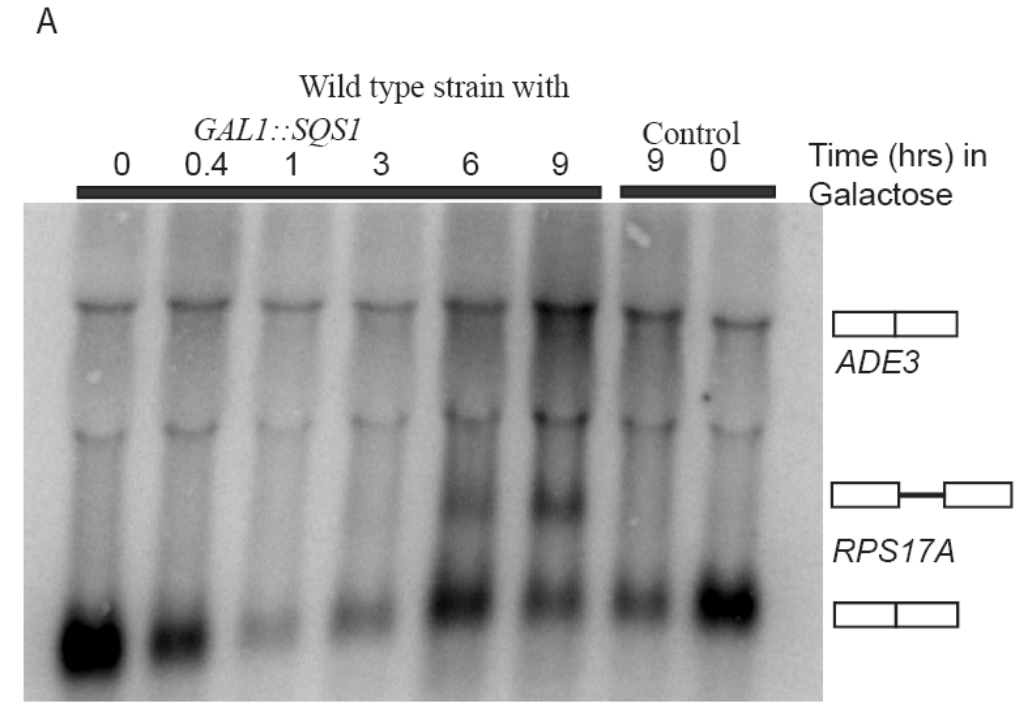
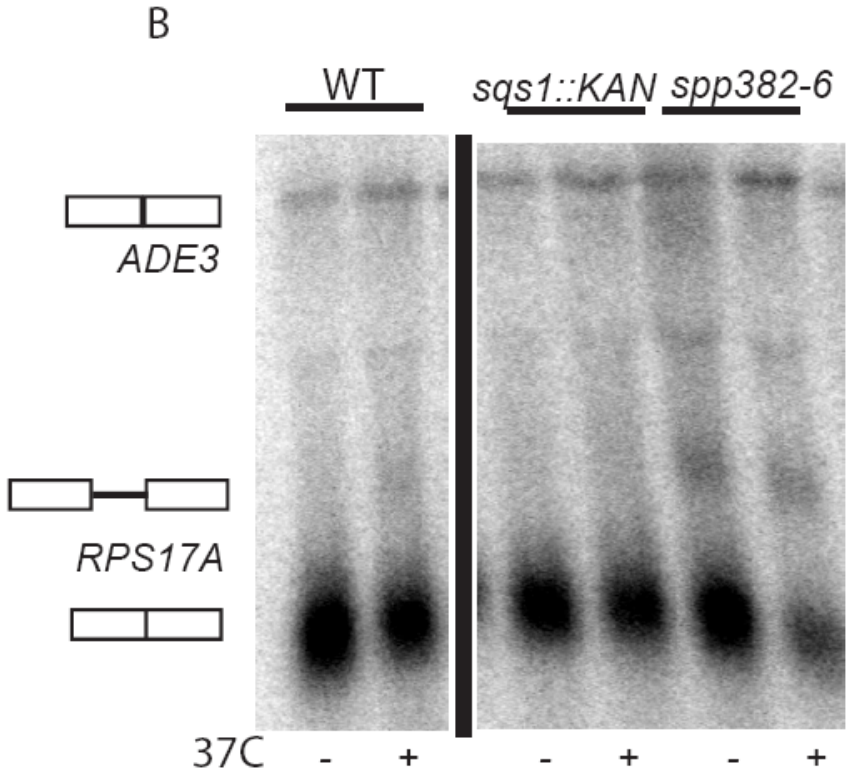

Section 1
(1) 1 10 20 38
YLR424w_Sc_664877 (1) TYGLGAALLSSLGLVACKCLGKDGSCITTPLETQSRPM
SQS1 (1) NENIGRRMDEKLGNKSGECIGIQGKCISRHTFRKFKKN
Luca15_Hs_1244404 (1) HSNLGNKMIQAMGREGSGLGRKCQCLIAPEAQVRLK
CC1442.13c_Sp_379 (1) NNGKGKQLEEMGRSRGKGLGSENQGMVPPVVAVVKNN
F4F15.230_At_4678941 (1) ADBWGHKLLSKMGKREGEGIGSSRKGMADLIMEGDVKT
Consensus (1) IG KLL MGWK G GLG GITDPI A VK
Section 2
(39) 39 48
YLR424w_Sc_664877 (39) HNVGLGMFSN
SQS1 (39) R-SGIRHSES
Luca15_Hs_1244404 (39) G-AGLGAKGS
CC1442.13c_Sp_379 (39) K-QGLH----
F4F15.230_At_4678941 (39) NNLGVGASAP
Consensus (39) AGLG

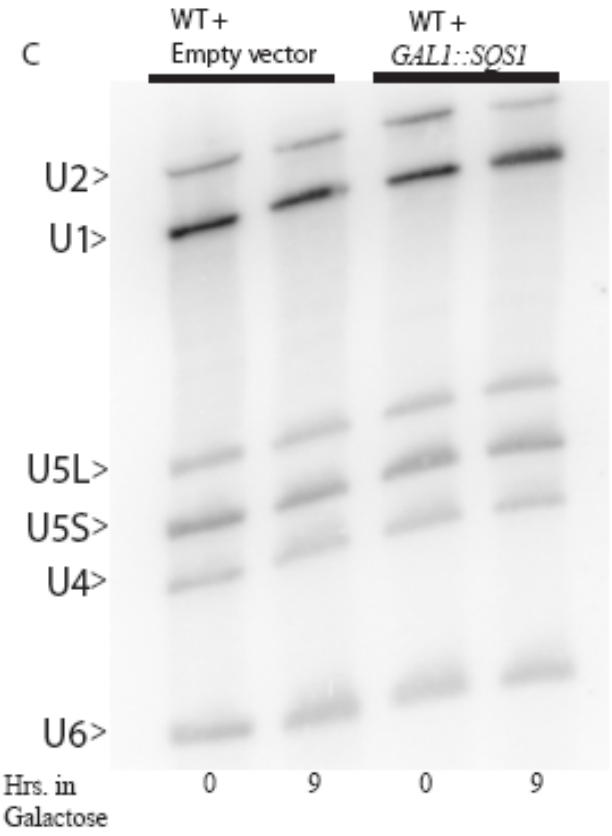
FIG.3.28 . Overexpression of Sqs1p impairs splicing. A. Northern analysis of splicing inhibition with wild type yeast transformed with the nutritionally regulated *GAL1::SQS1* gene before and up to 9 hours after transcriptional induction by galactose. Yeast that express a negative control vector (*GAL1::CYC1*) were assayed in parallel after growth in glucose (T=0) or galactose (T-=9). The positions of the intronless *ADE3* mRNA and the *RPS17A* pre-mRNA and mRNA are shown to the right.

B. Northern analysis of *sqs1::KAN,* a strain with chromosomal deletion of *SQS1* along with a wild type strain and *spp382-* grown either at room temperature (-) or at 37 °C (+).The positions of the intronless *ADE3* mRNA and the *RPS17A* pre-mRNA and mRNA are shown to the left

C. Northern analysis of the RNA from the overexpression experiment described above (A) assayed for snRNA stability. The positions of snRNAs are indicated to the right.


A
Wild type strain with
GAL1::SQS1
Control
0 0.4 1 3 6 9 9 0
Time (hrs) in Galactose
ADE3
RPS17A


B
WT
sqs1::KAN spp382-6
ADE3
RPS17A
37C
- + - + - +


C
WT +
Empty vector
WT +
GAL1::SQS1
U2>
U1>
U5L>
U5S>
U4>
U6>
Hrs. in
Galactose
0
9
0
9

Sqs1p interactions with Spp382p and related factors.

Three proteins, Spp382p, Cwc23p and Sqs1p were identified in this study as having genetic or biochemical links to Prp43p. I investigated interactions with this protein set by a directed two-hybrid study using full length proteins. As shown before (Figure 3.18), Spp382p interacts with Cwc23p. Sqs1p was found to interact with Spp382p but not with Cwc23p. Intriguingly, I could not generate a double transformant of Sqs1p and Prp43p, indicating that simultaneous expression of both factors as full length Gal4p fusion proteins is deleterious to the cell. Consistent with previously published reports (Tsai, Fu et al. 2005), Spp382p was observed to interact with the Prp43p DExD/H-box protein in the two hybrid study. No interaction was detected between Cwc23p and Prp43p by this assay (Figure 3.29).

As overexpression of Sqs1p impairs *spp382p* mediated suppression of *prp38-1,* I tested if absence of *SQS1* had any effect on growth of the *prp38-1* mutant. *SQS1::KAN* neither suppressed nor exacerbated growth of the *prp38-1* mutant (Figure 3.30).

FIG 3.29. Two Hybrid interactions of Sqs1 with Spp382p and related factors. Serial dilutions of the reporter yeast strain containing the indicated two hybrid vectors was spotted on with medium lacking adenine (-Ade, glu) or lacking histidine and supplemented with 20mM 3-aminotriazole (-His, glu +20 mM 3AT) and grown at 30 °C for 3 days. It should be noted that the figure is a composite of multiple plate cultures incubated at the indicated temperatures for identical periods of time.

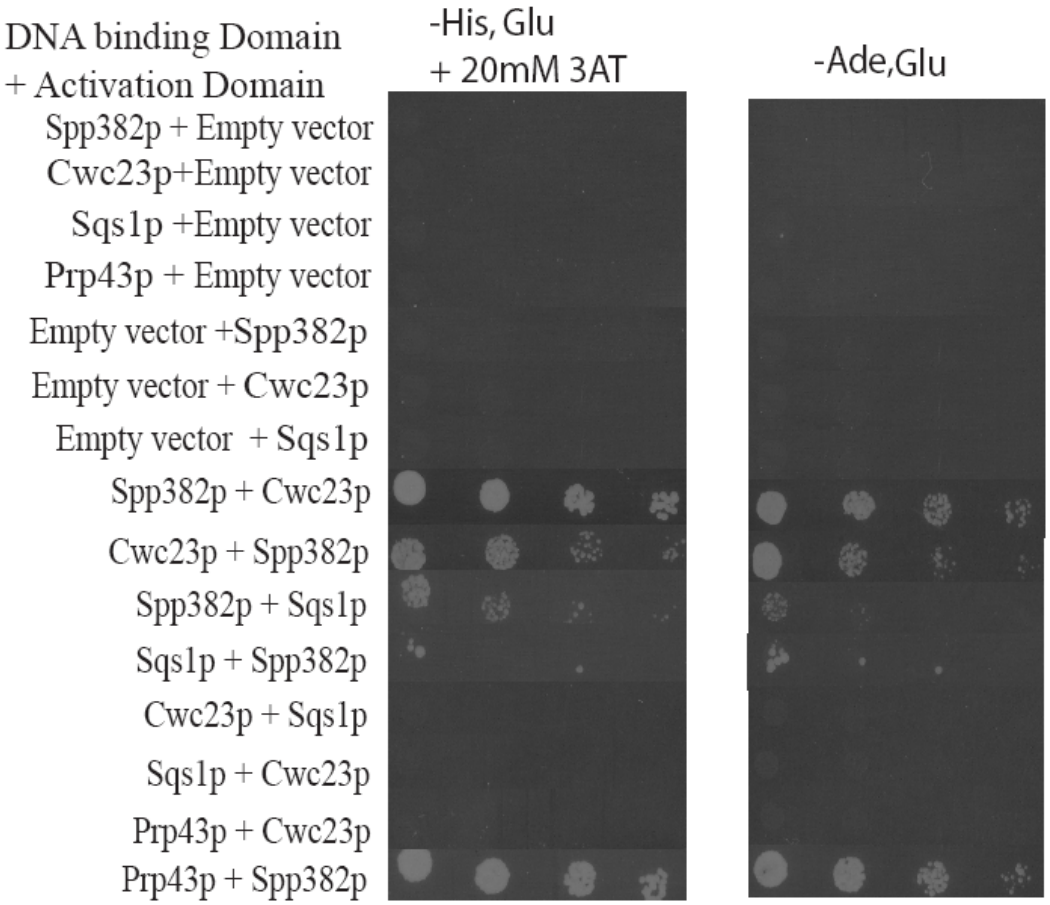
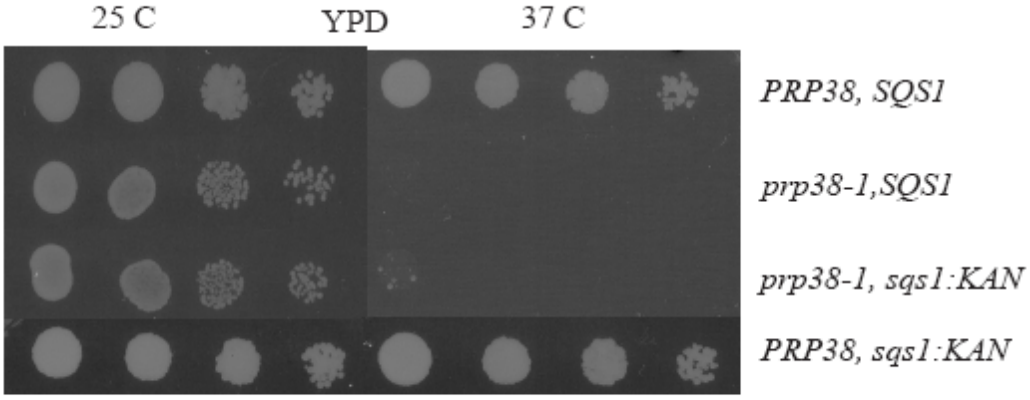

DNA binding Domain
+ Activation Domain
-His, Glu
+ 20mM 3AT
-Ade,Glu
Spp382p + Empty vector
Cwc23p+Empty vector
Sqs1p +Empty vector
Prp43p + Empty vector
Empty vector +Spp382p
Empty vector + Cwc23p
Empty vector + Sqs1p
Spp382p + Cwc23p
Cwc23p + Spp382p
Spp382p + Sqs1p
Sqs1p + Spp382p
Cwc23p + Sqs1p
Sqs1p + Cwc23p
Prp43p + Cwc23p
Prp43p + Spp382p

FIG 3.30. Sqs1 null allele does not suppress *prp38-1.* Serial dilutions of the indicated strains were spotted on the YPD plate and incubated at 25 °C for 4 days and 37 °C for 2 days.



CHAPTER 4.

DISCUSSION

The spliceosome is a complex, dynamic ribonucleoprotein (RNP) complex that undergoes numerous conformational changes during its assembly, activation, catalysis and disassembly. Many of these conformational changes are stimulated by the eight spliceosomal DExD/H box proteins at the expense of ATP. While needed for the spliceosome cycle, surprisingly little is known of how spliceosomal DExD/H proteins are recruited, the molecular details of target association, what governs the temporal restriction of their enzymatic activities, or even the primary targets of ATP-dependent function (e.g., RNA unwinding or protein displacement). In contrast, the spliceosome assembly defects associated with the loss of each activity is well documented. In their landmark studies on the Prp16p DExD/H-box protein, Burgess and Guthrie proposed a model in which the fidelity of correct branchpoint selection is coupled to ATP hydrolysis (Couto, Tamm et al. 1987; Burgess, Couto et al. 1990; Burgess and Guthrie 1993). More recently, Prp22p has been proposed to act in a similar manner during the 3' splice site selection. Both results illustrate what might be considered a basic gatekeeper feature of DExD/H-box ATPases in toggling alternative conformational states. Here, the ATP hydrolysis by each of the DExD/H proteins would drive the spliceosome cycle forward to the subsequent step and assure fidelity in splicing when coupled with a discard pathway for the removal of flawed complexes (Brow 2002; Query and Konarska 2004; Konarska and Query 2005). Key to the underlying "kinetic proofreading" mechanism is the existence of a

discard pathway to remove defective complexes. This discard pathway would insure against faulty and potentially deleterious splicing events as well as promote the recycling of limiting spliceosomal factors. While the components are unknown, such a discard pathway has been speculated to act with DExD/H box proteins throughout the spliceosome cycle as a quality control system for splicing complex integrity (Konarska and Query 2005).

The underlying hypothesis of my research is that a spliceosomal discard pathway exists in yeast and acts with the DExD/H box-proteins to promote faithful splicing. My dissertation studies used a genetic approach to identify putative components of this pathway and employed genetic, molecular, and biochemical methodologies to investigate the contributions of the identified factors to pre-mRNA splicing. This project was initiated with a genetic modifier screen that isolated trans-acting suppressors of the *prp*38-*1* mutant. Our previous studies provided strong evidence that *prp38-1* mutant yeast produced complete but kinetically impaired spliceosomes (Blanton, Srinivasan et al. 1992; Xie, Beickman et al. 1998). I proposed that the *prp38-1* suppressors would likely fall into two groups, 1) suppressors that improve splicing directly and 2) suppressors that disable the discard pathway and thereby give more time for the "slow" spliceosomes to work. Both predictions were supported by my observations. For the first class, many intragenic suppressors were recovered. These were noted but not chosen for further study since our primary interest was in extragenic events.

I propose that the mutation recovered within the uncharacterized open reading frame, *YLR424w*, is characteristic of the second type of suppressor, namely a suppressor that indirectly improves splicing through impairment of the discard pathway. I have registered this gene with the Saccharomyces Genome Database as *SPP382*, for the second suppressor of *prp38-1* (that is suppressor *prp38-1* #2). The amino acid sequence of Spp382p provides little insight into its function although this is clearly an evolutionarily conserved protein (Figure 3.2) that associates with components of the yeast and mammalian spliceosomes (Gavin, Bosche et al. 2002; Hazbun, Malmstrom et al. 2003; Deckert, Hartmuth et al. 2006; Collins, Kemmeren et al. 2007; Wang, Q., K. Hobbs, et al. 2003). This protein does contain a G-patch motif, a ~ 48 amino acid motif containing six highly conserved glycine residues, implicated in RNA binding. Other G-path proteins in yeast include *SPP2, PXR1, SQS1* and an unknown open reading frame, *YLR271w.* Spp2p, like Spp382p, has been implicated in recruitment of a DExD/H factor, Prp2p. Pxr1p is implicated in rRNA and snoRNA maturation and the other two (Sqs1p and Ylr271w-p) are largely uncharacterized.

We previously observed the *YLR424w* –encoded protein as Ccf8p in a proteomics study of the yeast spliceosome (Wang, Hobbs et al. 2003). This earlier work used a late-acting splicing factor, Clf1p, in an affinity selection scheme to identify subunits of the mature spliceosome. At least two distinct multisubunit complexes were affinity purified with Clf1p, the mature or postcatalytic spliceosome containing late-acting splicing factors and the U2, U5 and U6 snRNAs and the RNA-free Prp19-complex (NTC) that acts late in the

assembly pathway and is released from the spliceosome after catalysis (Tsai, Chow et al. 1999; Chen, Yu et al. 2002; Wang, Hobbs et al. 2003). Of the ~30 proteins characterized, Ccf8p was unique in that it was the only factor preferentially enriched in the NTC fractions, prompting our suggestion that Ccf8p may be an important factor in NTC-spliceosome dynamics (Wang Hobbs etc, 2003). As discussed below, I believe that the results of my studies and other recent observations support this conclusion.

Though essential for cell survival, nothing was known about the cellular role of Spp382p in splicing at the time of my investigation. To begin to address this, I mutated conserved residues based on sequence homology. Mutating conserved residues in the G-patch either results in cell death (*spp382-9* or *10*) or impairs cell growth (*spp382-2 or 3*), indicating that this domain is essential for the protein's function. In general, mutations that affect growth also impair splicing and accumulate precursor mRNA at restrictive temperature, indicating that this protein is essential for the first transesterification reaction in vivo.

I found that extracts made from cells that were metabolically depleted of Spp382p (*spp382-4* grown in glucose) block spliceosome assembly prior to the addition of the tri–snRNP, i.e. at the pre-spliceosome stage. While this suggests a role in stable recruitment of the U4/U6.U5 tri-snRNP particle, we were not able to efficiently complement this defect by exogenously added Spp38p, leaving open the possibility that the initial Spp382p depletion resulted in the destabilization or parallel depletion of other factors acting at this stage in splicing.

Some of the mutants (*spp382-1, -3, -5* and -*6*) also accumulate excess amounts of the excised intron, as shown when probed with actin probe. Accumulation of intronic RNA is seen with mutants that play a role in spliceosome disassembly, like the 2'-5' phosphodiesterase that linearizes the lariat intron, Dbr1p, or the DExD/H protein involved in intron release and spliceosome disassembly, Prp43p (Chapman and Boeke 1991; Arenas and Abelson 1997), suggesting a role for *spp382* in disassembly of the splicing apparatus. Indeed it is quite possible to interpret the block in spliceosome assembly seen with metabolically depleted extracts as indirect and resulting from impaired recycling of splicing factors necessary for the first step of splicing. Extracts prepared from yeast cells metabolically depleted of Spp382p by transcriptional repression of a *GAL1* fusion allele also showed little or no tri-snRNP, increased amounts of the U4/U6 di-snRNP particle and what could be a U2. U5.U6 snRNP (see band labeled * in figure 3.10). Based on total RNA pools, spliceosomal snRNA stability does not appear to be reduced in cells metabolically depleted for Spp382p as it is seen with the inactivation of certain other splicing factors (e.g. Blanton et al., 1992). When the snRNP assembly is monitored using native gels, the depleted extract shows an aberrant, slowly moving complex above the tri-snRNP. A lesser amount of this complex can be seen with functional extracts, consistent with the possible super-accumulation of snRNP particles in a spliceosome like complex under Spp382p-limiting conditions. In brief, my results show that Spp382p depletion in vivo does affect

the tri- snRNP assembly, possibly because of impaired disassembly of the spliceosome.

Chen and co-workers showed that Spp382p is necessary in vitro for intron release from the mature spliceosome by the DExD/H helicase, Prp43p (Tsai, Fu et al. 2005). While the mechanism is not known, Spp382p and Prp43p were shown to interact by the two-hybrid method, supporting speculation that Prp43p may act as a recruitment factor for Prp43p (Tsai, Fu et al. 2005). While my data is consistent with such a model, I believe that other models may also explain these results and the other published work. For instance, it is possible that Spp382p acts more directly in Prp43p activation or substrate presentation.

The role of Prp43p in the dissociation of the late-stage spliceosome is consistent with a similar activity in the proposed discard pathway. Since, at least in vitro, Prp43p activity requires Spp382p, *spp382* mediated suppression of splicing factor mutations might reflect lowered Prp43p activity. Martin et. al. showed that the ATPase activity was necessary for the Prp43p in vivo function and a threshold activity of the protein was essential for viability (Martin, Schneider et al. 2002). I tested the ability of three such viable *prp43* mutants with successively lower ATPases activities to suppress *prp38-1.* In keeping with my assumption, I find that the *prp43* mutants suppress the *prp38-1* defect. Importantly, the degree of suppression was inversely proportional to the residual ATPase activity of the mutants characterized by Martin et. al. (Martin, Schneider et al. 2002). This leads us to porpose a model in which defects in spliceosome assembly, like those caused by *prp38-1,* prompt Spp382p-mediated disassembly

of the defective complex via Prp43p. In a simple model of splicing (Figure 4.1), newly synthesized pre-mRNA is either spliced or degraded, with the splicing fate favored under optimal conditions. We propose that under normal conditions, conformational changes coincident with mRNA formation allow Spp382p to stimulate Prp43p recruitment or activation and prompt intron release. When splicing is slowed by a mutation, the splicing complex may be dissociated before the completion of splicing and the pre-mRNA degraded or reassembled into a new spliceosome. I propose that under conditions of limited Spp382p activity the discard pathway slows and allows increased opportunity for splicing to occur with kinetically impaired splicing mutants such as *prp38-1*.

One prediction of this model is that *spp382* mutant suppression should not be limited to *prp38-1* but extend to other (although not necessarily all) mutations in the splicing apparatus. Indeed, I found in addition to *prp38-1*(G66D), *spp382* mutations suppress a second *prp38* allele (C87Y) as well as mutations in *prp8-2* and *prp19-1*. In addition, I recently obtained evidence that *spp382-1* can partially suppress a weak mutation in the pre-mRNA branchpoint region (UCCUAAC, HZ3 figure 3.16) and at the 3' splice site showing that the suppression may extend to substrate-level mutations and includes mutants defective in either transesterification step in splicing (data not shown). Several other splicing factor mutants that I assayed were not suppressed (*prp4-1, rds3-1, prp39-1, prp16-1* and *prp22-1*). The reasons for lack of suppression are not known but might include, for instance, the mutant forming a completely inactive spliceosome which would fail to proceed to the next step.

Defects in the splicing complex assembly such as those resulting from the *prp38-1* or *prp8-1* mutations might allow for premature Spp382p association or activation, resulting in spliceosome disassociation, likely via Prp43p. Consistent with this, immune precipitation studies with a TAP-tagged Spp382p indicates that this protein recovers an unusual defective splicing complex in which the 5' exon is dissociated. Such defective complexes are known to occur spontaneously in vitro and we showed that the levels of defective complexes increase with reduced 5' exon length. Parker and colleagues have provided evidence that such defective intermediates of splicing also form in vivo on natural substrates (Hilleren and Parker; 2003). While clearly showing Spp382p association, at this point we do not know whether exon loss promotes Spp382p binding or simply exposes the TAP epitope on a complex where Spp382p was already present. In addition, while it is clear that the TAP epitope on Spp382p does not significantly inhibit splicing in vivo or in vitro, I cannot rule out a weak defect that might contribute to formation of the 5' exonless complexes that were recovered. Intriguingly, while Spp382p binds spliceosomes that lack the upstream exon, Prp8p interacts with this upstream exon before lariat formation (Teigelkamp, Newman et al. 1995; Teigelkamp, Whittaker et al. 1995) and likely assists in its retention afterward (see Grainger and Beggs 2005).

Several additional lines of evidence support the model of cooperative Spp382p-Prp43p function in splicing. For instance, Spp382p and Prp43p interact via two hybrid analysis (this study and Tsai, Fu et al. 2005). In addition, I found that the *prp43R424A* mutation (Martin, Schneider et al. 2002) is synthetically

lethal with *spp382-4* and *spp382-6* alleles consistent with an exacerbation of the splicing defects. Also, I find that overexpression of Prp43p is sufficient to impair the *spp382-1* mediated suppression. One possible explanation for this last observation could be that the elevated Prp43p levels compensate for possible lower affinity mutant *spp382-1* gene product (hence restoring the turnover activity by mass action). Finally, our lab recently showed that partially purified Spp382p-complex from yeast extracts, in vitro, dissociates the snRNP-complete spliceosome in an ATP-dependant manner. In contrast, the U1/U2 containing prespliceosome present in the same mixture is fully resistant to dissociation (Rymond, unpublished). While at this point the assay is not selective for "defective" spliceosome, the observations are consistent with Spp382p association with late-stage splicing complexes and the stimulation of a turnover activity.

The sedimentation profile of the released intron complex (Martin, Schneider et al. 2002) is comparable to that of the nineteen complex (NTC) fractions enriched with Spp382p (Wang, Hobbs et al. 2003), consistent with at least transient association of Spp382p with late splicing complexes. The Cheng lab showed that the affinity purified complex containing Spp382p (called NTR complex containing Prp43p and Ntr2p along with Spp382p) is capable of disassembling the spliceosome, separating the U2, U6 U5- NTC and the lariat intron in vitro (Tsai, Fu et al. 2005). The Beggs lab recently confirmed that presence of Spp382p is required for the Prp43p to bind to the post-catalytic spliceosome (Boon, Auchynnikava, et al. 2006). Data from the Luhrmann lab

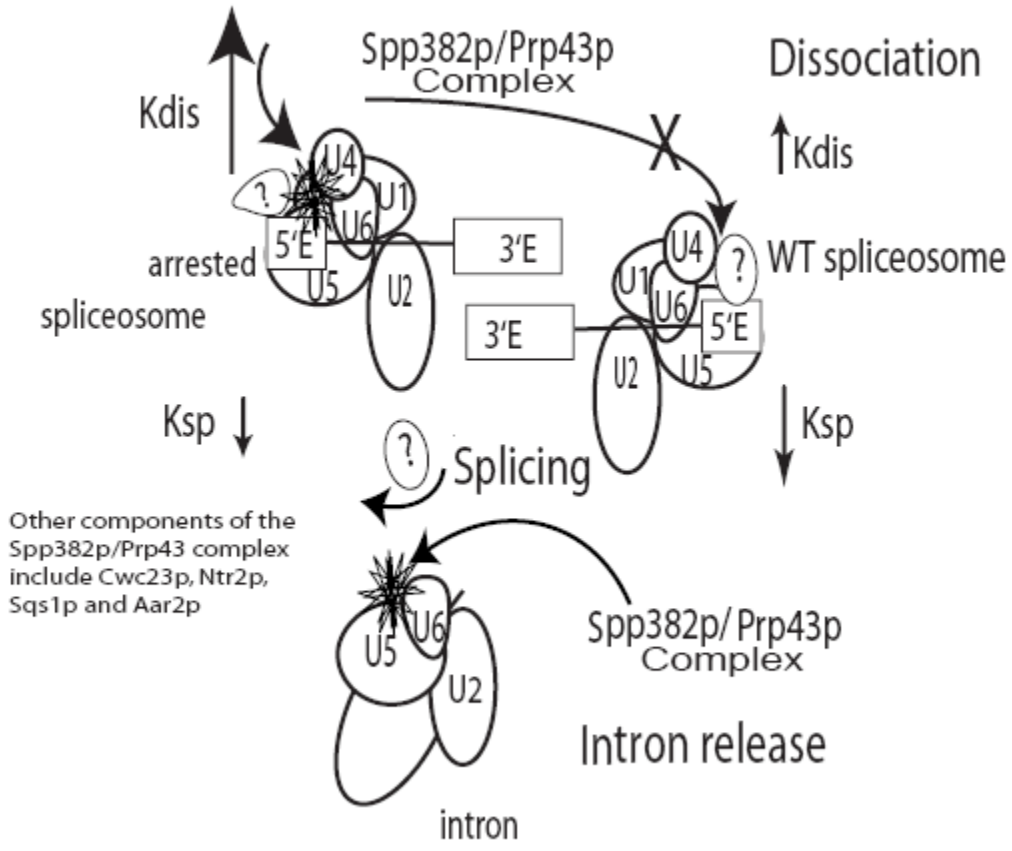
suggest that, in vivo, the NTC binds the spliceosome before the activation of the spliceosome and remains with the U5 snRNA after the completion of the catalytic reactions and disassembly of the spliceosome (Makarov, Makarova et al. 2002). Similar results have been observed in yeast (Tarn, Lee et al. 1993; Chan, Kao et al. 2003). Although NTC factors act earlier in splicing as reported by our lab (Wang, Hobbs et al. 2003). This 35S U5-NTC snRNP complex that exists after splicing has to be disassembled, presumably also by Spp382p dependant Prp43p activity. Indeed, we see a small amount of U5 snRNA with purified Spp382-TAP (Figure 3.9 C). This is consistent with the presence of a U5 snRNA containing post-spliceosomal complex (Makarov, Makarova et al. 2002). Prp8p, a U5 snRNP protein, cross links to the 5' splice site and is implicated in tethering the 5' exon for subsequent steps (Teigelkamp, Newman et al. 1995; Teigelkamp, Whittaker et al. 1995; Grainger and Beggs 2005). Both Spp382p and Aar2p, another U5 snRNP protein were recently found to be enriched in a complex containing mutant version of Prp8p (Boon, Auchynnikava et al. 2006).

A mutant allele of *aar2*, *aar2-D281N,* was also recovered as a suppressor of *prp38-1.* Aar2p is a component of the 16S U5 snRNP along with Prp8p, Snu114p and core Sm proteins. The 16S U5 snRNP co-purifies with U1 snRNP and this disnRNP particle has been implicated in 5' splice site recognition (Gottschalk, Kastner et al. 2001), suggesting an early co-operative role for U5 snRNP in the spliceosome assembly, particularly the 5'SS selection. Aar2p, also is required for the recycling of splicing factors. Splicing proceeds normally with depletion of Aar2p in vitro, unless "multiple turnover" of the spliceosome is

required. Under these conditions, extracts depleted of Aar2p fail to splice, indicating that Aar2p is necessary when splicing depends on snRNP recycling (Gottschalk, Kastner et al. 2001). Splicing factors are recycled to sustain multiple rounds of catalysis required for the processing of the abundant intron-bearing pre-mRNAs. Hence sequestering factors in dead end spliceosomes has dire effects on growth. We speculate that lowering but not abolishing the activity of a spliceosome recycling factor allows stalled but partially active spliceosomes additional time to splice. The published data are consistent with such a function for Aar2p but it is unclear for now whether Aar2p acts via Prp43p- mediated disassembly of defective spliceosomes.

The overexpression of several genes was found to reverse the salutary effects of diminished Spp382p activity on splicing mutants. While proteins linked by genetics or proteomics to Spp382p or Prp43p (i.e., Cwc23p, Sqs1p) likely enhance the recruitment or function of the proposed dissociation complex, it is unclear how the remaining effector genes act. In principle, any condition that enhances spliceosome disassembly should reduce *spp382-1* effectiveness in the *prp38-1* suppression assay. One can imagine, for instance, that excess levels of an early acting splicing factor (e.g., Prp39p (U1 snRNP), or Prp9p (U2 snRNP)) might prompt dissociation of a late-acting factor through competition for a common protein or RNA binding site. On the other hand, a protein like Dhh1p

FIG 4.1. Model of spliceosome integrity (SPIN) surveillance and intron release. The model proposes that Spp382p serves a role both in the release of correctly spliced intron and in the dissociation of defective spliceosomes. Under normal conditions, Spp382p binds (or is exposed on) the spliceosome concomitant with the changes bought about by the mRNA being release. This event precipitates Prp43p recruitment and intron release. When splicing is slowed by a mutation (e.g. *prp38-1, prp8-1,* or loss of 5' exon), this allows for premature Spp382p association (or exposure), resulting in spliceosome disassociation via Prp43p. By extension, decreased Spp382p or Prp43p activity is likely to extend the spliceosome stability and therefore the likelihood of processing by a partially active complex. Here, the oval bearing the question mark represents a factor that normally restricts Spp382p binding (or access) until completion of splicing or complex distortion by mutation.


Spp382p/Prp43p
Complex
Dissociation
Kdis
Kdis
U4
U1
U6
5'E
3'E
arrested
spliceosome
U5
U2
U4
U1
U6
5'E
3'E
U2
U5
WT spliceosome
Ksp
Ksp
Splicing
Other components of the
Spp382p/Prp43 complex
include Cwc23p, Ntr2p,
Sqs1p and Aar2p
U5
U6
U2
Spp382p/ Prp43p
Complex
Intron release
intron

may act via a completely different mechanism through premature pre-RNA nuclear export or decay (Coller and Parker 2005)

The *spp382-1* mutation resides within the proposed Ntr2p binding site region defined by two hybrid analysis (Uetz, Giot et al. 2000; Ito, Chiba et al. 2001; Chan, Kao et al. 2003)**.** Here, it is possible that overexpression of Ntr2p compensates for a weakened interaction between the two proteins to enhance spp382-1p activity on the *prp38-1* background. This also would explain how overexpression of Ntr2 also suppresses the modest *spp382-1* growth defect. While challenging, elucidation of the various *spp382-1* suppression blocking activities acting through gene overexpression may provide insight into the processes that influence spliceosome homeostasis. Recently, our lab looked at the effect of the overexpression of these proteins had on splicing in the wild type strain (data not shown). We found that overexpression of Prp8p, Sqs1p, Cwc23p and Spp382p impair splicing while overexpression of other factors did not impact splicing, at least for the *RPS17a* gene. However, we looked at only one time point after galactose induction (6 hours), which may have been insufficient to see the effect with the rest of the proteins.

Just as seen with Prp43p overexpression, two other proteins that co-purify with Prp43p (Sqs1p/Ynl224c-p, a G patch protein and Cwc23p, a Dna J like protein) also inhibit the growth of the spp382-*1, prp38-1* double mutant suppressed strain when overexpressed. It should be noted that neither one of these genes significantly alter growth of wildtype yeast or the *spp382-1* or *prp38-1* single mutants when grown on YP-galactose plates. *SQS1* overexpression has

been reported to impair cell cycle progression (Stevenson, Kennedy et al. 2001) and thus impair growth. I confirmed this observation when the assay was done on defined medium. Although, Sqs1p is not essential for either robust growth or splicing, I do find that overexpression of Sqs1p impairs both splicing and growth even in wild type strain. Sqs1p may increase the efficiency of splicing as many other non-essential splicing factors do (e.g. Mud2p association helps binding of the branchpoint binding protein). Hence it would not be required for splicing per se but overexpression might tie up binding partners essential for splicing and hence impair their normal function. For some inexplicable reason, we do see a difference in the rich vs. selective media.

Sqs1p, like the Prp43p activator, Spp382p and the Prp2p activator, Spp2p, is a G-patch protein. This observation raises the interesting question of whether Sqs1p might play a role similar to Spp382p in the recruitment or activation of DExD/H box proteins. The splicing defects when overexpressed along with two hybrid interaction with Spp382p suggest that it could do so. However, alternate models of Sqs1p function are possible- Sqs1p over expression might affect splicing indirectly (by inhibiting transcription of core splicing factors) or perhaps the “G-patch” of Sqs1p binds with lower affinity core splicing factors normally recruited via similar “G-patch” protein and renders them non-functional when present in excess amounts.

My proteomic study shows that Cwc23p co-purifies with Spp382p under high stringency, an interaction I confirmed by two hybrid analysis. Cwc23p, like Spp382p, is an essential protein whose role in splicing was not characterized

prior to this study. Mutational analysis of this protein shows that its J domain is important for function. My results demonstrate that Cwc23p mutants show impaired splicing and growth. Previous studies with DnaJ proteins in *E.coli* (Hsp40) and in yeast (Ydj1) (Wall, Zylicz et al. 1994; Wall, Zylicz et al. 1995; Becker, Walter et al. 1996; Yan and Craig 1999) show that mutations in the J-domain are either lethal or impair the activity of their Hsp70 partners. In addition, in vitro biochemical studies show that the J domain alone is sufficient to stimulate the ATPase activity of the Hsp70p similar to that observed with full length DnaJ protein (Wall, Zylicz et al. 1994). My results show that the DnaJ domain is also important for Cwc23p function and by extrapolation to the published studies, may play a role in regulating an ATPase in the spliceosome. This would be exciting because Cwc23p is the only known J domain protein in the spliceosome. Since, Cwc23p is found in proteomic complexes pulled down using Prp43-TAP (Gavin, Bosche et al. 2002; Tsai, Fu et al. 2005) or Spp382-TAP (this study) as bait, it is possible that Cwc23p modulates the Prp43 DExD/H -box protein.

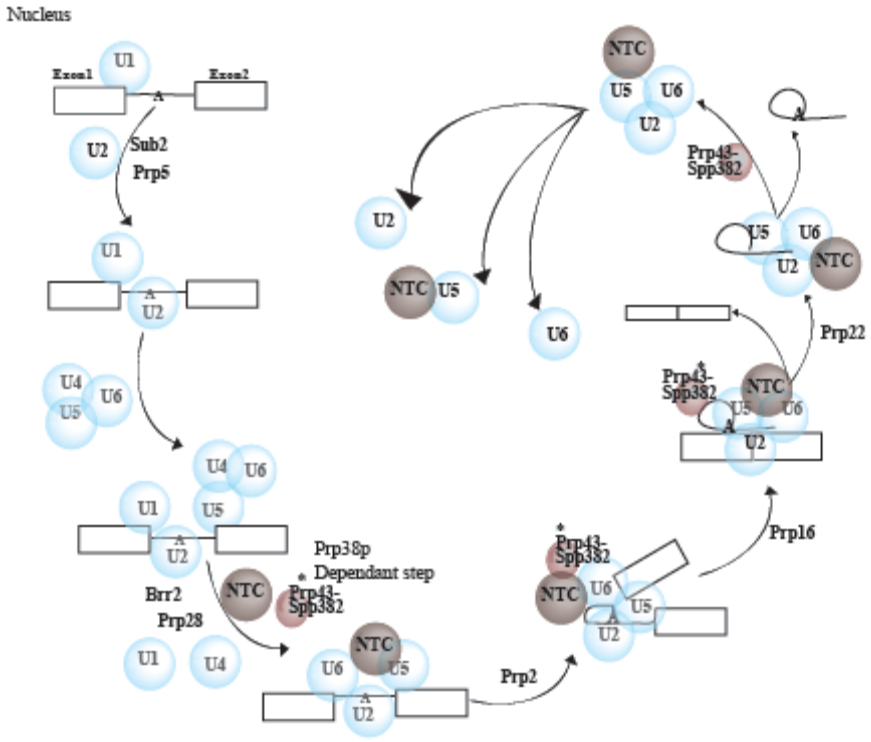
Primer extension analysis of mRNA isolated from several *cwc23* mutants reveals an inhibition of the first transesterification step in splicing when Cwc23p is inactivated. In addition some Cwc23p mutants also accumulate excess excised intron, like Spp382p or Prp43p, suggesting that this protein may play a role in recycling of splicing factors. I observed that the *cwc23* J-domain mutants fail to interact with the Prp43p binding partner Spp382p in the two hybrid assay. Although I find no evidence for Prp43p-Cwc23p interaction by two hybrid assay, over expression of Cwc23p in the *prp43-H218A* strain impairs cell growth. This

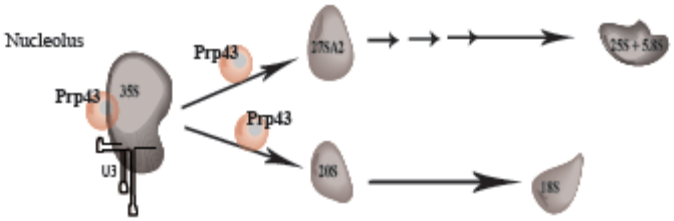
suggests that at least under the conditions tested, too much Cwc23p may dampen Prp34p activity. While I found no cases of suppression of other splicing mutants by the *cwc23* mutants, I found that the *prp8-2* mutation suppressed the growth impairment of the J domain mutants, *cwc23-1* and *cwc23-2*. Recently other *prp8* mutant alleles have been shown to suppress either the first step or second step splicing defects. The interpretation of the results from many genetic experiments is that Prp8p has a role in repositioning the pre-mRNA during the transition between the two catalytic steps of splicing (Liu, Query et al. 2007). In this light, *prp8* mutant could be relieving the *cwc23 -1* and *cwc23-2* blocked spliceosome.

In addition to its role in splicing, Prp43p plays a critical role in the rRNA processing, and is required for production of both the small and the large subunit rRNAs. Prp43p can be recovered with the 35S rRNA precursors and all the subsequent intermediates (Lebaron, Froment et al. 2005). It is also present in the very early pre -60S pre-ribosomal particles. *prp43* cold sensitive mutants show increased 35S pre-rRNA and depleted levels of 20S, 27S and 7S pre-rRNA precursors (Lebaron, Froment et al. 2005; Combs, Nagel et al. 2006; Leeds, Small et al. 2006). At the steady state, most Prp43p is found in the nucleolus, the site of rRNA biogenesis (Huh, Falvo et al. 2003). Since Prp43p plays a dual role in splicing and rRNA biogenesis, it is interesting to speculate on how the protein is functionally and physically partitioned. One clue arises from the observation that, in addition to binding the Spp382p splicing factor Prp43p interacts with Pxr1p, a second G-patch protein required for rRNA biogenesis. Our lab has

examined rRNA biogenesis in the *spp382* mutant background and could find no evidence for this protein acting in the rRNA pathway (data not shown), consistent with a specific role in splicing. Conceivably, the alternative G-patch proteins, Spp382p and Prx1p, bind Prp43p in a mutually exclusive fashion to direct the location and activity of Prp43p. This model is currently under investigation (see figure 4.2).

FIG. 4.2. Prp43p – Role in spliceosome and ribosome biogenesis. A schematic representation of (A) the Prp43p-Spp382p mediated steps (indicated with the pink circle) in splicing occurring in the nucleus. The * refers to conclusions based on the results of this study. (B) shows Prp43p dependant steps in rRNA biogenesis (orange circle) in the nucleolus.


Nucleus
Exon1
Exon2
U1
U2
Sub2
Prp5
U4
U6
U5
Brr2
Prp28
Prp38p
Dependant step
Prp43-
Spp382
NTC
Prp2
Prp16
Prp22


Nucleolus
Prp43
35S
U3
27SA2
20S
25S + 5.8S
18S

Future Directions-

The results presented here provide strong evidence for a Spp382-dependant spliceosome integrity (SPIN) complex acting in the discard pathway. While our data suggests that the SPIN complex acts to disassemble the spliceosome possibly at several stages (Prp38p, Prp8p or Prp19p dependant step), it is unclear how the complex recognizes defective substrates and what other components make up the SPIN surveillance complex. Based on the available genetic and biochemical data, we suspect that Spp382p interactions with Prp43p, Cwc23p, Ntr2p and perhaps Sqs1p may contribute to SPIN activity. However, it remains to be investigated if there are other proteins that also work with Prp43p-Spp382p at this surveillance function. Moreover, the role that Cwc23p and Sqs1p play in this complex remains to be understood.

How the cells target the defective complex and how the surveillance complex is kept in check to avoid inappropriate turnover of active spliceosome also remains to be addressed. Since the cell regulates protein activity by chemical modification (eg. phosphorylation, acetylation, ubiquitination etc), it would be interesting to see whether proteins involved in the SPIN complex are present with such modifying groups in vivo. It remains to be investigated whether SPIN surveillance, the turnover of postcatalytic spliceosomes, and Prp43p function in ribosome biogenesis (Lebaron, Froment et al. 2005; Combs, Nagel et al. 2006) are accomplished by entities of identical or overlapping protein compositions. Since Prp43p interacts with two G-patch proteins, Spp382p (which is essential for splicing) and Pxr1p (involved in rRNA biogenesis), it is interesting

to speculate whether these two G-patch proteins are responsible for partitioning Prp43p between the two separate complexes. Since both Spp2 and Spp382p, are now believed to be involved in recruiting the DExD/H box proteins to the spliceosome, it will be important to learn how substrate selection is determined by these and the other known yeast G-patch proteins (Pxr1p, Sqs1 and Ylr271w-p).

Appendix 1.

Spp381p – Prp38p –Snu23p form a stable heterotrimeric complex.

Prp38p is a small (28 kDa), highly acidic tri-snRNP specific protein that is required for spliceosome maturation (Blanton, Srinivasan et al. 1992);(Xie, Beickman et al. 1998). Prp38p mutants fail to unwind U4/U6 helices, an important step for activation of spliceosomes. Prp38p is unlikely to be a helicase (based on absence of canonical helicase motifs) but it may act to recruit or activate one. The Prp38p mediated release of the U4 snRNA is not well understood.

To begin our study of Prp38p function, we wanted to investigate what this protein binds in vivo. We metabolically labeled yeast with 35S-methionine and cysteine and purified proteins from a Prp38-TAP strain and an untagged control. Purification with Prp38p-TAP pulled showed a heterotrimeric complex. The Prp38p protein is associated with two proteins, Spp381p, previously identified as a dosage suppressor of *prp38-1*, (Lybarger, Beickman et al. 1999) and a small protein, the same molecular weight as previously identified Snu23p, a tri-snRNP protein. To see whether this assignment is true, I purified the complex using Prp38-TAP, Spp381-TAP, and Snu23-TAP tagged strains. All three strains recover the same three proteins (i.e., Prp38p, Prp381p, Snu23p), as indicated by band shifts. The asterix indicates the position of proteins with a residual TAP tag. To avoid recovery of complex spliceosomes, two step TAP selection was performed at 450 mM NaCl. The proteins were resolved on a polyacrylamide gel and, where possible, the tentative protein assignments (based on observed sizes

and previous studies) were confirmed by western analysis by using the anti-CBP and the anti-Prp38p antibody (data not shown).

Consistent with the affinity purification results, the three proteins also interact by two hybrid assay as shown in table A1.1.

FIG A1. Prp38p-Spp381p-Snu23p form stable heterotrimeric complex. 35S-labeled proteins from Spp382-TAP, Prp38-TAP, Spp381-TAP or an untagged yeast strain were isolated by two step TAP selection at 450 mM NaCl and resolved on a polyacrylamide gel. The protein assignments were confirmed by western analysis. The CBP indicates the residual tag that remains after TEV cleavage.

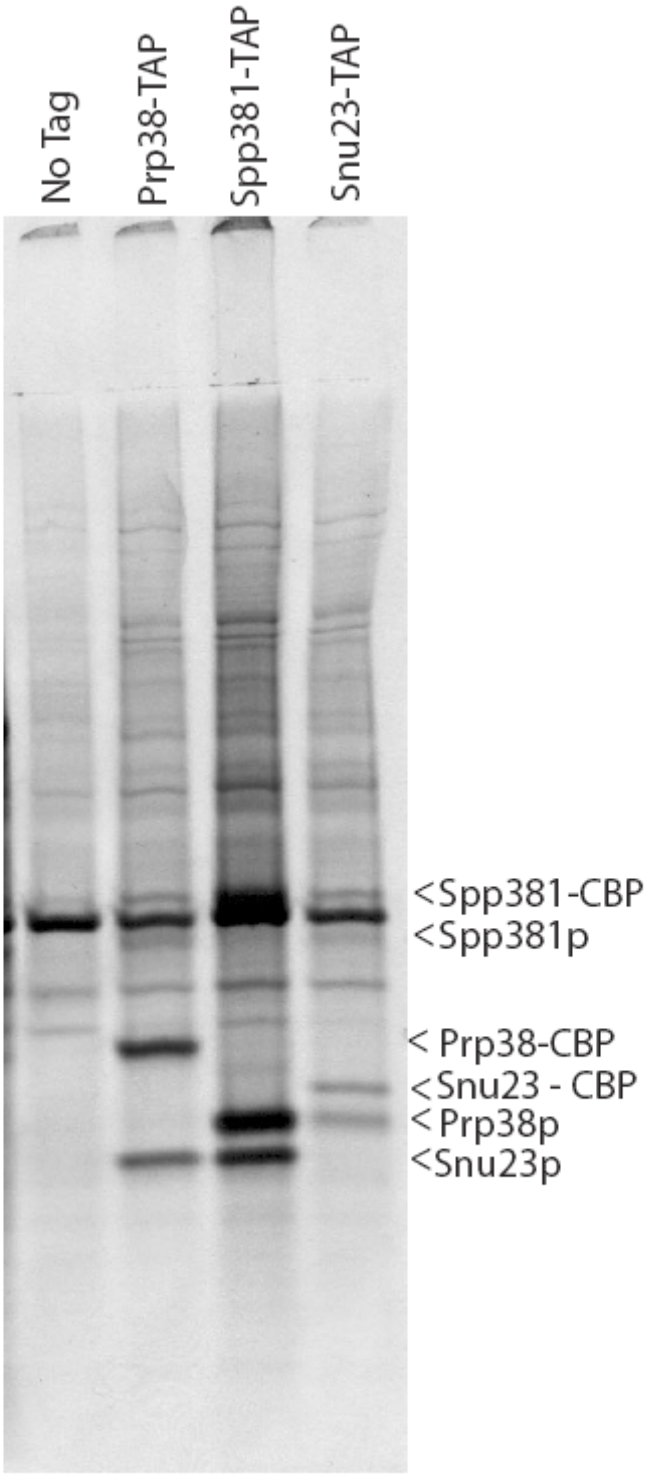

No Tag
Prp38-TAP
Spp381-TAP
Snu23-TAP
<Spp381-CBP
<Spp381p
< Prp38-CBP
<Snu23 - CBP
<Prp38p
<Snu23p

Table A1.1. Two hybrid interactions. The yeast strain PJ69-4a, was co-transformed with the indicated Gal4p fusion products and growth assayed on medium lacking histidine and supplemented with 3 aminotriazole.

pACT	pAS2	Interaction
Prp38	Spp381	Yes
Prp38	Snu23	Yes
Snu23	Spp381	Yes
Spp381	Snu23	Yes

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Aravind, L. and E. V. Koonin (1999). "G-patch: a new conserved domain in eukaryotic RNA-processing proteins and type D retroviral polyproteins." Trends Biochem Sci **24**(9): 342-4.

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